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organotypic 3-dimensional *in vitro* models

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I. List of Abbreviations:

2D	Two-dimensional
(k)Da	(kilo) Dalton
3D	Three-dimensional
ADME	Absorption-Distribution-Metabolism-Elimination
AFP	Aequorea-derived fluorescent protein
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CAF	Cancer-associated Fibroblast
Cis-DDP	Cis-Diamminedichloroplatinum II
DAPI	4',6-Diamidin-2-phenylindol
DNA	Deoxyribonucleic Acid
DT	Doubling Time
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGFRI	Epidermal Growth Factor Receptor Inhibitor
EMT	Epithelial-mesenchymal Transition
FADD	Fas-associated Death Domain
FFG	Österreichische Forschungsförderungsgesellschaft
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
GLUT-3	Glucose Transporter-3
GPCR	G-protein Coupled Receptor
HBSS	Hank's Balanced Salt Solution
HCS	High-content Screening
HIF-1,2	Hypoxia-inducible Factor-1&2
HRP	Horseradish Peroxidase
HTS	High-throughput Screening
IRES	Internal Ribosomal Entry Site
IF	Immunofluorescence
NGAL	Lipocalin 2
mAb	monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
mCFP	Monomeric Cyan Fluorescent Protein

MCTO	Multi Cellular Tumor Organoid
MET	Mesenchymal-epithelial Transition
miRNA	micro-Ribonucleic Acid
NASA	National Aeronautics and Space Administration
NCI60	National Cancer Institute 60
nHDF	Neonate Human Dermal Fibroblast
NID	New Investigational Drug
NSCLC	Non-small Cell Lung Cancer
ORF	Open Reading Frame
pAb	polyclonal Antibody
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline + Tween
PDGF	Platelet Derived Growth Factor
pEGFR	phosphorylated Epidermal Growth Factor Receptor
PGA	Polyglycolic Acid
PI3K	Phosphoinositide-3-Kinase
PLA	Polylactic Acid
PLGA	Poly(lactic-co-glycolic) Acid
poly-HEMA	poly 2-Hydroxyethyl Methacrylate
PS	Phosphatidylserine
PTM	Post-translational Modifications
RBp	Retinoblastoma-associated Protein
RCCS	Rotary Cell Culture System
RNA	Ribonucleic Acid
RT	Room Temperature
RTKI	Receptor Tyrosine Kinase Inhibitor
SA- β -Gal	Senescence-associated β -galactosidase
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SMWC	Small Molecular Weight Compound
TSP-1	Thrombospondin-1
TVDT	Tumor Volume Doubling Time
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
wtGFP	wild-type Green Fluorescent Protein

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1 Summary

English| To overcome potential shortcomings of classical monolayer cell culture and animal models in drug discovery, complex three-dimensional cell-based cancer models entered the stage in the last years. These artificial tissue surrogates serve as suitable ecological niches for a multitude of cell types. They allow mimicking the pathophysiological complexity and cellular heterogeneity of cancer *in vivo*. Therefore they might lay the foundation for a robust and reliable *in vitro* cancer model. In cell-based three-dimensional models it is possible to tailor the appropriate microenvironment for many individual tumors. However, many research efforts are still necessary in order to fully integrate 3D models into the drug discovery strategies of pharmaceutical industry.

In this work we established robust and standardized methods to generate 3D cancer models on the basis of the human tumor spheroid methodology. Spheroids are spherical cell aggregates that tend to form under defined experimental culture conditions. Since each cancer cell line required individual conditions to form spheroids we used different techniques including Rotary Cell Culture System (RCCS), the hanging drop and Methylcellulose-based methods as well as non-adherent surfaces (Hydrocell™, poly-HEMA coating) to generate spheroids. Due to recent advances in the field of biomaterials individually tunable matrices have been developed to provide the embedded spheroids with tailored microenvironment. We embedded spheroids in natural (Matrigel™, Collagen type 1) and synthetic (HyStem™, HydroMatrix™) extracellular matrix (ECM) scaffolds. This facilitated the generation of heterotypic co-cultures consisting of tumor and stromal cells.

We stably transduced lung cancer cells and primary human fibroblasts to express fluorescent proteins (EGFP, mCFP, J-Red) which allowed discriminating between the different cell types. We used these cells to establish defined co-cultures of lung cancer spheroids with human lung fibroblasts that may allow studying tumor-stroma interactions. Such co-cultures are considered to be a promising tool in tumor biology

for deciphering the effects of tumor-stroma interactions on drug response and drug efficacy in the future. We already established an experimental workflow for the automatized generation of spheroids and organoids that might be amenable to high-throughput drug screening (HTS).

To characterize the cell physiology of organotypic 3D models in more detail we performed Affymetrix Gene Chips analyses. We could demonstrate that organotypic cultivation in 3D ECM matrices (Matrigel™/collagen) substantially altered the gene expression profile of cancer cells. Furthermore using defined phospho-protein antibody arrays we revealed differential phosphorylation patterns when cells were cultivated in 3D. In particular, we could show that epidermal growth factor receptor (EGFR) is significantly hyperphosphorylated in the tested lung cancer cell lines cultivated in 3D. In line with these findings the 3D models exhibited a significantly increased sensitivity to the targeted EGFR inhibitor Gefitinib (Iressa) while cancer cells cultivated in 2D were only weakly affected. In the clinic it could be demonstrated that the survival of lung cancer cells in cancer patients was strictly dependent on hyperactivated EGFR signaling (oncogene addiction). Moreover we could demonstrate that 3D models also exhibited different responses to the chemotherapeutic paclitaxel. In this case organotypic cultivation rendered the 3D cells more resistant to paclitaxel than in the 2D cultures. We suggest that new drugs that could have beneficial effects *in vivo* might be identified only in 3D model-based drug screenings. For the evaluation of drug efficacy within our 3D model, we established 3D cell-based assays for the detection of proliferation and apoptosis (AlamarBlue®, Caspase-Glo® 3/7, Annexin V). We used these assays to evaluate drug sensitivity of spheroids generated from four lung cancer cell lines.

Taken together, our data clearly demonstrate that cancer cell physiology and drug responsiveness might be clearly different in 2D and 3D cancer models. In the case of EGFR signaling only the 3D models exhibited a drug response that was comparable to clinical observations in cancer patients (oncogene addiction). However, much more work is clearly needed in order to fully exploit organotypic 3D models for basic and applied cancer research and personalized medicine.

Deutsch| Um potenzielle Defizite von konventioneller Einzelschichtkultur- und Tiermodellen in der Medikamentenentwicklung zu umgehen, wurden in den letzten Jahren komplexe drei-dimensionale zellbasierte Krebsmodelle entwickelt. Diese dienen als ökologische Nische für eine Vielzahl von unterschiedlichen Zelltypen. 3D Modelle erlauben es die pathophysiologische Komplexität und Heterogenität von Krebs zu imitieren. Daher könnten sie als Basis für robuste und verlässliche *in vitro* Krebsmodelle dienen. In zellbasierten 3D Modellen ist es möglich das geeignete Mikroumfeld für viele verschiedene Tumore maßzuschneidern. Nichtsdestotrotz ist noch viel Forschungsarbeit notwendig, um 3D Modelle in der Pharmaindustrie im Bereich der Wirkstoffentwicklung integrieren zu können.

In dieser wissenschaftlichen Arbeit haben wir robuste, standardisierte Methoden entwickelt, um 3D Krebsmodelle auf Basis der Tumorsphäroid-Methodik aufzubauen. Sphäroide sind sphärische Zellaggregate, welche sich unter definierten experimentellen Kulturbedingungen spontan formen. Da jede Zelllinie individuelle Bedingungen benötigt um Sphäroide zu bilden haben wir unterschiedliche Techniken benutzt. Darunter befinden sich das „Rotary Cell Culture System“ (RCCS), die „Hanging drop“ und die Methylzellulose-basierten Methoden, sowie nicht-adhärente Oberflächen (Hydrocell™, poly-HEMA Beschichtung). Aufgrund der Fortschritte auf dem Gebiet der Biomaterialien konnten bereits unterschiedliche Matrizen entwickelt werden, die in der Lage sind, den eingebetteten Sphäroiden ein maßgeschneidertes Mikroumfeld zu bieten. Wir haben Sphäroide in natürlichen (Matrigel™, Collagen Typ 1) und synthetischen (HyStem™, HydroMatrix™) extrazellulären Matrizen (ECM) eingebettet. Dies ermöglichte heterotypische Ko-Kulturen, die aus Tumor- und Stromazellen bestehen. Des Weiteren wurden menschliche Lungenzellen und primäre Fibroblasten stabil transduziert, sodass sie Fluoreszenzproteine exprimierten (EGFP, mCFP, J-Red). Damit konnten wir zwischen den unterschiedlichen Zelltypen unterscheiden. So konnten wir definierte Ko-Kulturen generieren, in welchen es möglich ist, Tumor-Stroma Interaktionen zu studieren. Dieses Modell ist ein vielversprechendes Werkzeug in der Tumorbilogie um die Auswirkungen der Tumor-Stroma Interaktionen auf die Medikamentensensitivität zu studieren. Wir haben bereits einen experimentellen

Arbeitsablauf für die automatisierte Generierung von Sphäroiden und Organoiden (eingebettete Sphäroide) definiert, der für Hochdurchsatz-Screenings (HTS) geeignet sein könnte.

Um die Zellphysiologie von organotypischen 3D Modellen genauer charakterisieren zu können, haben wir Affymetrix Gene Chip Analysen durchgeführt. Wir konnten so zeigen, dass die organotypische Kultivierung in 3D ECM Matrizen das Genexpressionsprofil der Krebszellen wesentlich veränderte. Mithilfe von Phospho-Proteom Antikörperarrays konnten wir signifikante Veränderungen in der Phosphorylierung wichtiger Proteine zeigen, sobald die Zellen in 3D kultiviert wurden. Im Besonderen betraf dies den EGF-(Epidermal Growth Factor) Rezeptor. Dieser wurde in den untersuchten Lungenkrebszelllinien, sobald sie drei-dimensional kultiviert wurden, hyperphosphoryliert. Dementsprechend sprachen die 3D Modelle wesentlich sensitiver auf die Behandlung mit dem EGFR Hemmer Gefitinib (Iressa) als Einzelschichtkulturen. In der klinischen Behandlung von Lungenkrebspatienten konnte gezeigt werden, dass das Überleben der Krebszellen stark von der Hyperaktivität des EGF-Rezeptors abhängig war (oncogene addiction). Weiters konnten wir zeigen, dass 3D Modelle ebenso auf das Chemotherapeutikum Paclitaxel anders reagierten. In diesem Fall waren die 3D-kultivierten Zellen resistenter gegenüber dem Therapeutikum als Einzelschichtkulturen. Daher wäre es durchaus denkbar, dass neue wirksame Medikamente nur in 3D-Modell-basierten Wirkstoff Screenings ihre günstige Wirkung offenbaren.

Um die Möglichkeit Therapeutika auf deren Wirksamkeit in den etablierten 3D Modellen zu testen, entwickelten wir Protokolle um zellbasierte Assays für Detektion von Zellproliferation und Apoptose durchzuführen (alamarBlue®, Caspase-Glo® 3/7, Annexin V). Mithilfe dieser Assays bestimmten wir die Medikamentenempfindlichkeit von Sphäroiden von vier Lungenkrebszelllinien.

Zusammengefasst konnten wir eindeutig zeigen, dass Zellphysiologie und das Ansprechen auf Wirkstoffe in 2D und 3D Krebsmodellen unterschiedlich sein kann. Im Falle vom EGFR-Signalweg konnten nur in den 3D Modellen die klinischen Beobachtungen bestätigt werden (oncogene addiction). Es sei jedoch darauf

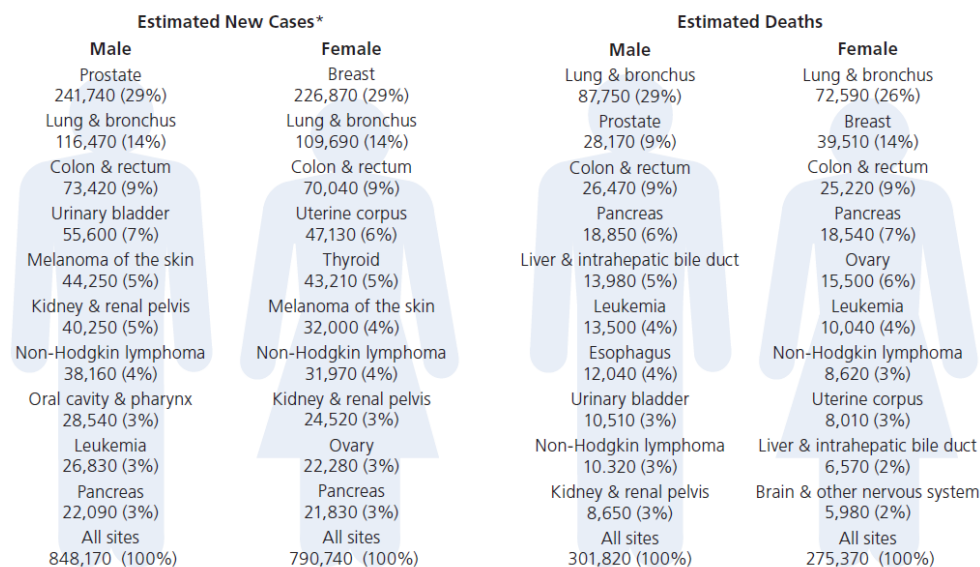
hingewiesen, dass noch viel Arbeit nötig ist, um das Potential organotypischer 3D Modelle sowohl für die Grundlagen- und angewandte Krebsforschung als auch für personalisierte Medizin vollständig ausschöpfen zu können.

2 Introduction

2.1 Development of Cancer and Cancer Progression

Even though more than seventy years have been elapsed since the first use of chemotherapeutics, cancer is still second most frequent cause of death worldwide accounting for 7.6 million deaths in the year 2008, outranged only by heart diseases. This number is predicted to rise to 12 million deaths per year until 2030 according to WHO Data and Statistics (New Cancer cases and deaths 2012, Figure 1). It is essential to extend our still poor knowledge about the initiation and driving forces of cancer in order to functionally understand and treat this complex disease.

Leading New Cancer Cases and Deaths – 2012 Estimates



*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder.

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Figure 1: New cancer cases and deaths in the USA in 2012.

The highest cancer incidence rates has prostate cancer in men and breast cancer in women, both accounting for 29% of all leading sites followed by lung cancer in both sexes. Nevertheless, lung cancer accounts for more deaths than any other cancer type regardless of gender. According to American Cancer Society an estimated 160,340 lung cancer related deaths (28% of all cancer deaths) are expected to occur in 2012.

In general cancer is a multifactorial disease which is characterized by deregulated cell proliferation and dissemination of altered cells in the organism. This is enabled by abnormal gene expression and aberrant gene function in these transformed cells.

All capabilities that a cancer cell acquires being necessary for tumor growth, progression, invasion and metastasis can be subsumed as hallmarks of cancer ([Hanahan D, Weinberg RA, 2000, 2011](#)).

2.1.1 Sustaining Proliferative Signaling

The probably most fundamental attribute of cancer cells is their ability to sustain continuous proliferation by overcoming cellular control elements by e.g. disrupting negative feedback mechanisms or up-regulation of growth promoting signals. Negative feedback loops are essential in normal tissue to ensure homeostasis of intracellular signaling by restraining various types of signaling pathways. Aberrations in negative feedback circuitry are frequently found in various malignancies since defects in this control element could enhance the proliferative capacity of tumor cells. In order to acquire and maintain the proliferative potential the activation of additional downstream pathways marks a key event in cancer progression. Homeostasis of cell number and tissue architecture is maintained in normal tissues by the controlled balance of growth-promoting signals that enable cell growth and cells' entry and progression through the cell cycle. These growth promoting signals are predominantly growth factors that bind receptor tyrosine kinase receptors on the cell-surface e.g. epidermal growth factor receptor (EGFR). In a majority of different cancer types sustained proliferation is maintained upon constitutive signaling through an activating mutation affecting mitogen-activated protein (MAP)-kinase pathway (~40% of human melanomas) and/or hyperactivation of phosphoinositide 3 (PI3)-kinase pathway ([Jiang and Liu, 2009](#)).

2.1.2 Evading Growth Suppressors

In order to maintain up-regulation of cell growth and proliferation cancer cells additionally adopt the ability to evade programs that negatively regulate cell proliferation and often depend on two canonical tumor suppressor proteins TP53 and RB (retinoblastoma-associated). Furthermore these are key proteins not only in the regulation of proliferation but also in the activation of senescence and apoptosis. Upon various mainly extra- but also intracellular signals RB functions as a

gatekeeper at the G1 to S phase restriction point and decides whether to proceed in the cell cycle or trigger senescence or apoptosis program ([Burkhart and Sage, 2008](#)). In contrast TP53 serves as a sensor to various mainly intracellular stress conditions and DNA damage and can consequently induce mitotic arrest or if the damage is irreparable can trigger apoptosis.

2.1.3 Resisting Cell Death

Since alteration of cellular circuitry and its resulting opportunity to evade growth suppressors, one key feature of cancer is the loss of TP53 tumor suppressor function, downregulation of pro-apoptotic factors such as Bax, Bim, and Puma or increase of expression of anti-apoptotic factors such as Bcl-2, Bcl-x_L ([Ren D et al., 2010](#)). In contrast to apoptosis where the cell shrinks to a small corpse followed by its consumption through neighboring cells the necrotic cells increase in size until they burst. During this process termed necrosis the cell debris, pro-inflammatory signals and bio-active regulatory factors are released into the local tissue microenvironment, a feature which is not observed in apoptosis or autophagy. As a consequence inflammatory cells of the immune system are recruited during necrotic events which can then promote the tumor actively in terms of growth, tumor progression and metastasis. More than that, other tumor stroma cells such as cancer-associated fibroblasts (CAFs) and cancer-associated macrophages (CAMs) are stimulated by the released factors such as interleukins to foster tumorigenesis and to evade cell death programs ([Hanahan D, Weinberg RA, 2011](#)).

2.1.4 Enabling Replicative Immortality

Since alteration of cellular circuitry and its resulting opportunity to evade growth suppressors, one key feature of cancer is the loss of TP53 tumor suppressor function, downregulation of pro-apoptotic factors such as Bax, Bim, and Puma or increase of expression of anti-apoptotic factors such as Bcl-2, Bcl-x_L ([Ren D et al., 2010](#)). In contrast to apoptosis where the cell shrinks to a small corpse followed by its consumption through neighboring cells the necrotic cells increase in size until they burst. During this process termed necrosis the cell debris, pro-inflammatory signals

and bio-active regulatory factors are released into the local tissue microenvironment, a feature which is not observed in apoptosis or autophagy. As a consequence inflammatory cells of the immune system are recruited during necrotic events which can then promote the tumor actively in terms of growth, tumor progression and metastasis. More than that, other tumor stroma cells such as cancer-associated fibroblasts (CAFs) and cancer-associated macrophages (CAMs) are stimulated by the released factors such as interleukins to foster tumorigenesis and to evade cell death programs ([Hanahan D, Weinberg RA, 2011](#)).

2.1.5 Inducing Angiogenesis

In order to be provided with sufficient nutrients and oxygen and to ensure appropriate removal of toxic metabolites and carbon dioxide a tumor needs vasculature. During tumor progression the generation of tumor blood supply is a rate-limiting step and therefore exceptionally essential in tumorigenesis. But since sufficient oxygen and nutrient supply can only be ensured for cells residing within ~100-200 μm of vasculature, angiogenesis is often persistently switched in most tumors ([Dang CV and Semenza GL, 1999](#)). Normally the formation of new blood vessels is mainly associated with embryogenesis. Therefore, the tumor must induce neovascularization to support tumor growth. This onset of angiogenesis in tumors is called the 'angiogenic switch' and can occur at any developmental stage during tumor progression depending on different varying requirements. It is known that the 'angiogenic switch' is controlled by angiogenesis inducers being mainly receptor tyrosine kinase ligands such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet derived growth factor (PDGF) and epidermal growth factor (EGF). On the other hand inhibitory molecules such as statins (angiostatin, canstatin or endostatin) and Thrombospondin-1 (TSP-1) counter angiogenesis by arresting an endothelial cell in a quiescent state ([Bergers B and Benjamin LE, 2002](#)). Due to an acquired imbalance of these mediators in the tumor the 'angiogenic switch' is kept at 'on'-position. Hence the tumor's blood supply allows tumor growth to proceed and ultimately also serves as a prerequisite for the onset of cancer metastasis.

2.1.6 Activating Invasion and Metastasis

A key moment of tumorigenesis is invasion and metastasis which is a multistage process during which malignant cells enter vasculature and spread from the primary tumor to distant sites in the body where they proliferate and form new tumor foci (Talmadge JE and Fidler IJ, 2010; Poste G and Fidler IJ, 1980; Figure 2A). Stephen Paget's 1889 proposal on the 'Seed and Soil Hypothesis' exemplified that the process of invasion and metastasis is dependent on both, immanent properties of the tumor cells and of the host response. This suggests that invasion and metastasis are not stochastic events but a complex sequence of events. Consequently metastasis is highly susceptible to any interference ultimately resulting in failure of forming secondary tumors. For this purpose carcinoma cells can exploit a multifaceted regulatory program termed epithelial-mesenchymal transition (EMT) to acquire attributes enabling invasion, apoptosis resistance and dissemination. EMT is governed by highly conserved effector molecules (Figure 2B) such as Wnts, fibroblast growth factors (FGFs), snails, Zeb1/2 and E-cadherin (Eger et al., 2004, 2005). The latter is known to be a master regulator maintaining adherens junctions, tight junctions and desmosomes of fully polarized epithelial cells and hence their basolateral and apical orientation. These cell-cell contacts impede the motility of normal epithelial cells. During the EMT program these epithelial markers are downregulated in concomitance with up-regulation of mesenchymal markers such as fibronectin, vimentin or smooth-muscle actin (Aigner K et al., 2007). The resulting phenotype acquires mesenchymal gene-expression patterns and properties which enable invasion into the tissue and blood stream and ultimately to form secondary tumor foci more efficiently (Thiery JP and Sleeman JP, 2006; Eger A and Mikulits W, 2005).

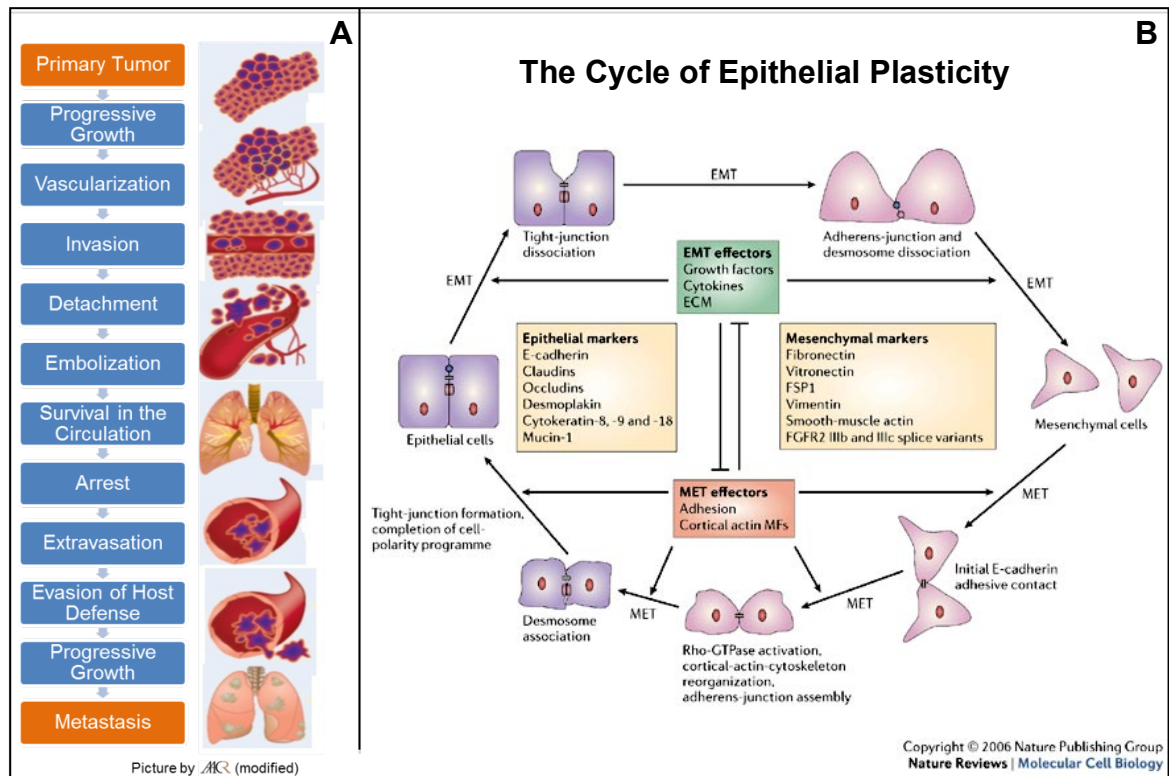


Figure 2: A| The Invasion-metastasis cascade.

Metastasis is a multistage process in which cancer cells disseminate from the primary tumor to form secondary tumor foci at the same or distant organs. This process consists of the sequential steps of local invasion into tissue, intravasation into blood vessels, survival in the circulation, extravasation from blood vessels into the tissue and colonization in other organ environments. Each selective step is potentially rate limiting and comprises stochastic elements which ultimately could impede or prevent completion of metastasis.

B| The cycle of epithelial-mesenchymal transition and its reciprocal process of mesenchymal-epithelial transition.

In this diagram the high plasticity of epithelial cells is depicted. Often during the initiation of metastasis carcinoma cells exploit the regulatory program of epithelial-mesenchymal transition (EMT) to gain cell motility which facilitate the evasion from the primary tumor. EMT and its reversed process are regulated by EMT and MET effectors respectively. A key event during EMT and MET is the regulation of cell-cell and cell-substrate anchors such as tight junctions, adherens junctions and desmosomes. ECM, extracellular matrix; FGFR2, fibroblast-growth-factor receptor-2; FSP1, fibroblast-specific protein-1; MFs, microfilaments.

Nevertheless at the site of metastasis carcinoma cells are then confronted with the absence of continuous signaling of EMT effectors which formerly triggered and maintained EMT at the primary tumor site. This may drive mesenchymal-epithelial transition (MET), a reciprocal program during which the expression of epithelial markers reconvenes resulting in the reversion of the cancer cells to a noninvasive state (Hanahan D, Weinberg RA, 2011).

This only outlined a number of the multifarious aberrations which result in disturbances of homeostasis paving the way for more than 100 distinct types and subtypes of cancer known today (Hanahan D, Weinberg RA, 2000). The path of cells on the way to malignancy can vary substantially which is reflected by the fact that histologically identical tumors may comprise completely different subsets of mutations. As a consequence the development of new potent anti-cancer therapies for a broad range of different cancer types is a challenging mission. Nevertheless, it is a straightforward approach to target the acquired cancer hallmarks in order to interfere with tumor growth and progression. An outline of potential anti-cancer agents which interfere with the cancer capabilities is depicted in Figure 3.

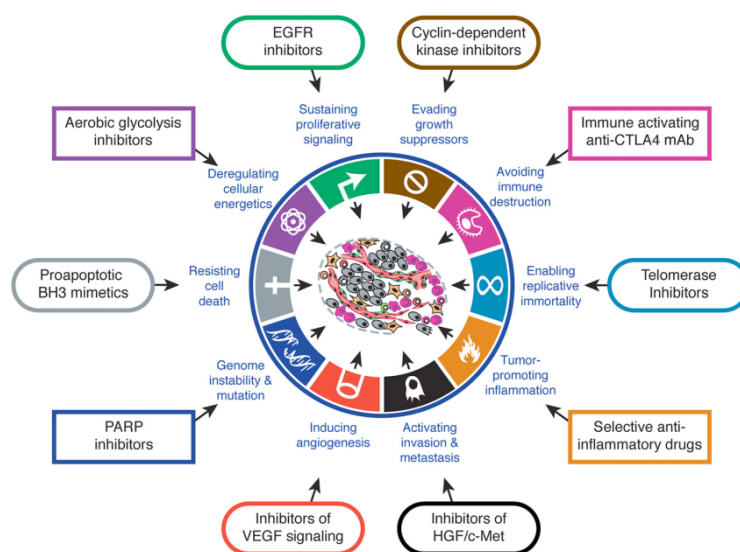


Figure 3: Examples of drugs targeting hallmark capabilities acquired by cancer cells during tumorigenesis.

Due to the fact that the cancer hallmark capabilities are required for tumor growth, progression, invasion and metastasis they serve as logical targets for anti-cancer drugs. Therefore several drugs targeting these hallmarks have been developed or are already in clinical use and show promising potential. Picture by Hanahan D, Weinberg RA, (2011) Hallmarks of cancer: The next generation.

2.2 Drug Discovery

2.2.1 Early Stages

Early Stages

Historically, pharmacological studies relied exclusively on animal tests to provide reliable indications of drug efficacy and safety. In 1959, William M. S. Russel and Rex L. Burch, two English scientists, carried out a study in which they systematically gathered data on animal experimentation. Upon their investigations Russel and Burch defined ethical aspects for the “development and progress of humane techniques in the laboratory” and initiated the **3Rs** movement to **R**eplace, **R**educe and **R**efine the use of animals in experimentation (Russel WMS and Burch RL, 1959). Due to strong international support for the 3Rs movement the focus was shifted from animal testing to the development of alternative *in vitro* techniques. Since 1996 the 3Rs are referenced in the “Guide for the Care and Use of Laboratory Animals” a 140-page document issued by the National Academy Press. The 3Rs movement paved the way for cell-based *ex vivo* drug discovery and soon the remarkable advantages of cell-based assays i.e. predictability, reproducibility, possibility of automation, reduction of costs and miniaturization implied that this tool is even more than an alternative to *in vivo* studies in early stages of drug discovery process. The large portfolio of cell-based assays includes a variety of assays that measure cell proliferation, toxicity, motility/invasiveness, activation of specific signaling pathways and changes in morphology (Michelini E et al., 2010). Due to their versatility these assays can be used in the drug discovery of various diseases.

2.2.2 Cancer Drug Discovery

A primary area of contemporary medical research is the discovery and development of new anti-cancer drugs. Several promising approaches such as targeted therapies, nanotechnology or gene therapy have been developed in the last decades to fight this heterogeneous disease. In 2008 more than 800 new anti-cancer agents were in phase I clinical trials certainly raising hopes to cure cancer. Nevertheless, anti-

cancer drug discovery in particular is fraught with obstacles such as lack of efficacy and safety of the drug. Due to that only ~10 percent of those 800 new anti-cancer agents will master the rocky road to registration and clinical utility, an expensive and more than a decade lasting endeavor with costs of US\$1 billion.

From Drug Target to Clinical Candidate The modern anti-cancer drug discovery process is the interplay between various disciplines including genetics, genomics and bioinformatics, cell and molecular biology, structural biology, pharmacology, pharmacokinetics and metabolism, medicinal chemistry, and experimental medicine (Neidle S, 2008). A typical anti-cancer drug discovery cascade is depicted in Figure 4.

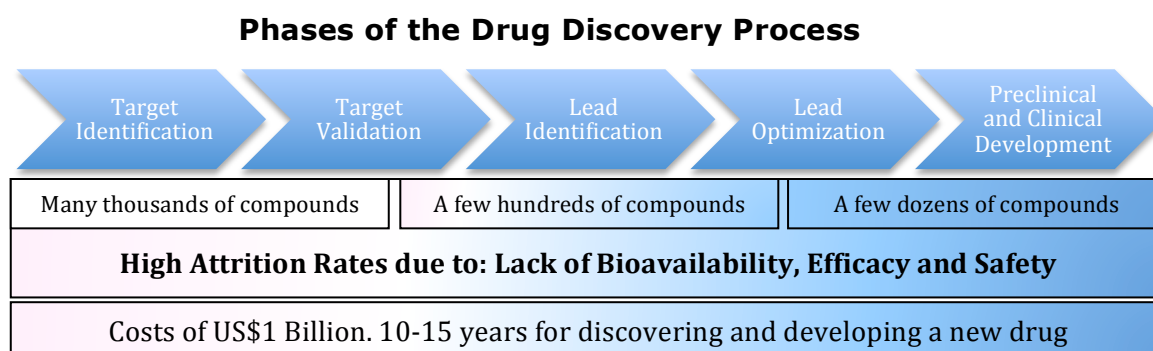


Figure 4: The drug discovery process.

Identifying and developing a potent drug is a process of sequential phases during which the candidate compounds are tested for their specific activity in a certain therapeutic context. During this process the number of drug candidates is continuously decreasing from many thousands of compounds (target identification) down to a few remnants in the preclinical and clinical development. The lack of bioavailability, efficacy and safety of drug candidates are the main reasons for the high attrition rates during the drug discovery process. Optimization of drug developmental processes including the establishment of better predictive models is therefore crucial for the reduction of failure rates in modern drug discovery. Only then the time for development of a highly potent drug and its associated costs of ~US\$1 billion could be reduced.

Starting from initial target identification and validation in biochemical screening assays (millions of compounds) using HTS a lead compound is being identified. The subsequent target modulation due to the desired cellular effect such as induction of apoptosis or growth inhibition is assessed *in vitro* using well characterized cell lines. Using high-throughput campaigns at this stage the ADME (i.e. absorption, distribution, metabolism, and elimination) properties of the investigational drug can be predicted for following *in vivo* tests. In order to give a profound prediction for the

drug's ability to traverse cell membranes, pKa, lipophilicity and aqueous solubility is determined. Thereafter, the demonstration of anti-cancer efficacy at atoxic doses of a few dozens of compounds is performed in animal studies. Prior to pre-clinical trials the evaluation on toxicology, manufacture and formulation are carried out in one rodent and one non-rodent species.

As depicted in Figure 4, a vast majority of new investigational drugs (NID) are already predestinated to fail in its infancy. In order to reduce high drop-out rates of NID Lipinski's 'rule of 5' is commonly considered in very early stages of drug discovery (Lipinski CA et al., 2001). This rule was derived from analyses showing that 90 percent of orally absorbed drugs had certain physical characteristics:

- Molecular weight smaller than 500 Da
- An octanol-water partition coefficient (lipophilicity) smaller than 5
- Fewer than 5 hydrogen-bond donors
- Less than 10 hydrogen-bond acceptors

Since orally absorbable drugs are generally preferred it is very common that screening libraries contain many compounds that obey the "rule of 5". Therapeutics which are exceptions to Lipinski's rule are e.g. antibiotics and cardiac glycosides since they are compound classes that are substrates for biological transporters (Lipinski CA et al., 2001).

In the past decades the discovery and validation of new anti-cancer drugs in preclinical studies was limited by the fact that most conventional cancer models did not faithfully recapitulate the complex disease *ex vivo*. In most cases cancer cell lines (e.g. NCI60 & CMT1000 platforms) or primary cancer cells that were isolated from whole tumor samples were simply propagated on two-dimensional (2D) synthetic substrata such as glass or plastic that force cells to adjust to artificially flat and rigid surfaces. Due to those conditions they lacked essential properties found *in vivo* (e.g. response of the three-dimensional microenvironment present in tissues)

and hence failed to serve as reliable predictors of drug efficacy prior to animal testing (Sharma SV et al., 2010). Therefore there is the necessity for alternative testing methods and innovative models including cell aggregates and tissue slices which recapitulate the complex pathophysiology of cancer more reliable *in vitro*.

Tissue engineering, the establishment of artificial multicomponent three-dimensional tissues, approaches the *in vivo* situation where 2D cell culture fails and hence may be a powerful tool for modern drug screening campaigns. These cell based surrogates can comprise organ-specific properties and therefore may serve as a suitable ecological niche for individual cell types. The artificial tissue surrogates could be used for studying disease pathogenesis and developing new *in vitro* assays for anti-cancer drug screening (Bhadriraju K et al., 2002).

At present *in vitro* generated microtumors that consist of several cell types and that are embedded in different extracellular matrices (ECM) might serve as novel and innovative drug discovery models. They might span the gap between 2D cultures and *in vivo* studies to screen for new anti-cancer drugs. The engineered microtumors mimic the complex pathology of cancer more closely and consequently may predict *in vivo* drug efficacy more reliable than 2D cultures.

2.3 Cell-based Assays in Drug Discovery

To exploit the full potential of the above depicted *in vitro* models to evaluate the pharmacology of candidate anti-cancer agents a large panel of cell-based assays has been developed. It is more than 60 years now since the first human cancer cell line (HeLa) was developed at Johns Hopkins Medicine (Baltimore, Maryland, USA). HeLa cells marked the beginning of established cell lines and enabled the development of drugs against numerous diseases (Lucey BP et al., 2009). Today a large panel of easy-to-cultivate immortalized cell lines is available with cell history, growth behavior and genomic information provided from a multitude of repositories such as the American Type Culture Collection (ATCC). Therefore these characterized human tumor-derived cell lines serve as ideal candidates for functional screens in anti-cancer drug discovery.

2.3.1 NCI60 and CMT1000 Cell Line Panels

The development of the first high-throughput cancer cell line screening program of its kind the US National Cancer Institute 60 anti-cancer drug screen (NCI60) started in 1986 (Figure 5). The 60 cell lines constituting this panel were selected due to various features including acceptable growth characteristics and sterility (Shoemaker RH et al., 2006). Cell-based assays, miniaturization in the form of microtiter plates and automated liquid handling is largely attributable to this scientific achievement whose development and operation has spanned over 20 years. In the late 80s and 90s of the last century 60 different cancer cell lines seemed to be an adequate number comprising the NCI60 panel. The reason for that was that the cancer treatments using predominantly nonspecific cytotoxic agents resulted in high rates of clinical responses. It was suggested that 6-9 human tumor-derived cell line representatives for each type of cancer would be sufficient to estimate the individual drug response (Shoemaker RH et al., 2006; Sharma SV et al., 2010). Nevertheless, the clinical activity of modern targeted anti-cancer agents is often limited to only a small subgroup of patients which ultimately indicated the need for larger cell panels. The

CMT1000 platform, launched in 2006, included 1,200 cell lines in the year 2010. By using this cancer cell line-based screening platform it was possible to demonstrate that the clinical responses to targeted inhibitors could be traced back to genomic aberrations to a remarkable degree (Sharma SV et al., 2010). This finding indicates the necessity of cell-based *in vitro* assays in the drug discovery process.

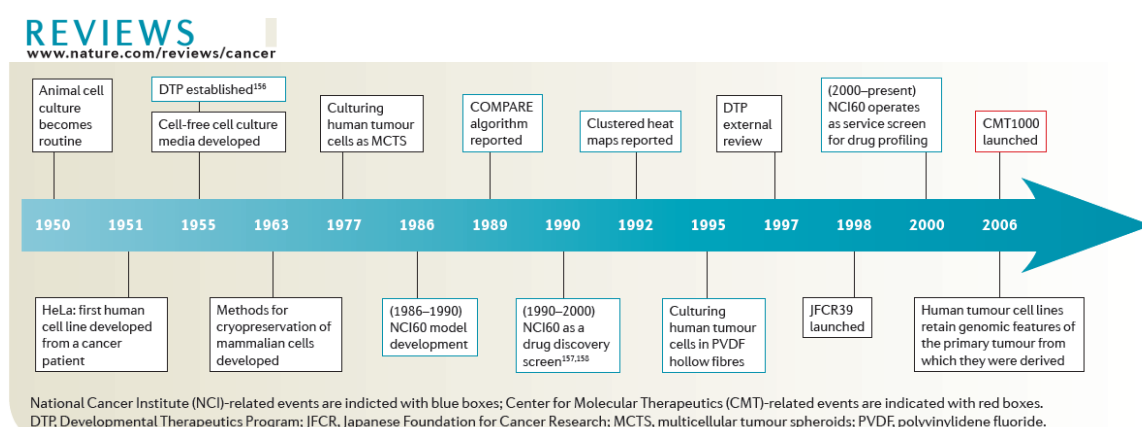


Figure 5: History of the development of cell-line platforms for evaluating anti-cancer agents.

Since decades the notable success of anti-cancer drug discovery and development is highly dependent on human tumor-derived cell line platforms such as NCI60 and the recently launched CMT1000. Picture by Sharma SV *et al.*, 2010, Nature Reviews Cancer (10):241-253.

2.3.2 High-throughput Screening (HTS) and High-content Screening (HCS)

Due to the multiplicity of detection technologies that have been developed in the past decades the potent discipline of cell-based high-throughput screening (HTS) could be established around 1990. In these campaigns large libraries of around 100,000 to 2 million differently small molecules are screened to allow the generation of lead compounds against specific molecular targets in cancer cells (e.g. kinases, receptors, transporters). Prior to 1989 it was virtually unfeasible to screen this tremendous number of compounds for their biological activity. Combined with the rapid progress in molecular genetics (e.g. expression of receptor subtypes in cells normally lacking their expression) and the general advantages of *in vitro* assays (i.e. predictability, reproducibility, possibility of automation) HTS campaigns are straightforward tools in modern pharmaceutical industry (Lipinski CA et al., 2001).

In order to exploit the inherent capabilities of HTS various automated systems have been developed. The Array Scan[®] (Cellomics, Pittsburgh, PA, USA) was the first system which integrated microscopy, digital imaging and data processing within a single platform (Giuliano KA, 1997). This versatile imaging platform was designed for high capacity analyses (high content screening, HCS) for drug discovery campaigns using cellular assays in 96- and 384-well plates. By using fluorescent probes (i.e. antibodies chemically coupled to low-molecular-weight fluorogenic dyes; fluorogenic proteins such as GFP genetically fused to desired cellular target) for HCS it is possible to measure a multitude of cellular features at once. The obtained data can deliver solutions for target validation and lead optimization and therefore improve the drug development process. Nevertheless, while low-molecular-weight dyes coupled to proteins fail to visualize intracellular targets (inability to enter living cells) the use of reporter proteins has certain limitations, as well. The high molecular mass of fluorogenic proteins can promote aggregation which subsequently leads to a quench of fluorescent signal. Therefore, instead of using fluorogenic proteins also synthetic membrane-permeable fluorophores (e.g. 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein) are used. When these fluorophores encounter a specific recombinantly expressed protein in the target cell it binds to it and starts to fluoresce (Griffin BA et al., 1998). This was only an outline of how data for drug discovery studies are assessed in HTS today. The portfolio of assays to evaluate a new investigational drug (NID) is virtually inexhaustible today.

2.3.3 Cell-based Assays

The development of rapid, reliable and cost-efficient high-throughput screening assays is of substantial importance during the process of drug discovery and development. By exploiting the large portfolio of cell-based assays in the very early stages of drug discovery data about the drug-candidate leads' biological activity, toxicological profile, biochemical mechanisms, and off-target interactions can be assessed. Recently, cell-based screening methods were used to evaluate drug related effects on several target classes such as G protein-coupled receptors (GPCR), ion channels and transporters, kinases and phosphatases, as well as

nuclear transcription factors (Michellini E et al., 2010). The therapeutic efficacy of a candidate anti-cancer agent can be evaluated by the means of its ability to induce the reversion of the acquired cancer hallmark capabilities i.e. sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis (Figure 3). Hence a large panel of different cell-based *in vitro* assays has been established in the past.

Apoptosis Assays Due to functional studies it is commonly accepted that apoptosis, the process of programmed cell death, serves as a natural barrier to tumorigenesis since it is a regulatory program to ensure tissue homeostasis (Lowe et al., 2004). The uncontrolled proliferation of cancer cells can be maintained by bypassing apoptosis. This is often achieved due to mutations found in apoptosis related circuitry and relaying proteins such as TP53, Bcl-2, Bcl-x_L, Bax, Bim, Puma (Hanahan D, Weinberg RA, 2000, 2011). Therefore this circuitry is a target for many anti-cancer drugs in order to regain command over the apoptosis machinery and hence to induce apoptosis in cancer cells ultimately. Apoptosis assays in which apoptotic cells can be quantified accurately are therefore of prime importance in order to evaluate the efficacy of a candidate anti-cancer drug (Berg et al., 2007). Due to that a large panel of cell-based apoptosis assays is available today which ideally quantify apoptotic cells rapidly, reliable, safe, sensitive, simple and cost efficient (O'Brien J et al., 2000).

Since both, the intrinsic (cytochrome C) and the extrinsic (Fas-associated Death Domain, FADD) apoptotic pathway ultimately culminates in the activation of apoptosis-associated proteases called caspases a common target to quantify apoptotic cells are caspases (commonly caspase-3, 7, 8, and 9, Alberts et al., 2008). Since caspases, or cysteine-aspartic-proteases, this feature is commonly utilized in apoptosis assays to determine apoptotic events. For this many apoptosis assays use compounds that are cleaved by caspases. These compounds are conjugated with a detectable molecule to assess data colorimetrically, luminometrically or

fluorometrically. Commonly used apoptosis assays are Promega's CytoTox-Fluor™ cytotoxicity assay which detects minute amounts of extracellular caspase activities fluorometrically and Promega's Caspase-Glo® 3/7 assay which luminometrically (luciferase) detects caspase 3/7 activity in a cell lysate. Both assays can be multiplexed to measure more than one parameter per well at once (acc. to Promega, Mannheim, Germany).

Another possibility to determine apoptotic events is the apoptosis associated switch of phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane. Under non-apoptotic conditions PS is exclusively located in the inner leaflet of the membrane. In the early stages of the apoptotic program PS is exposed on the outer leaflet as a result of decreased aminophospholipid translocase activity and activation of a calcium-dependent scramblase. In a physiological context this exposure is important for phagocytes to recognize and remove apoptotic cells (Fadok VA et al., 1998). Since Annexin V, a human vascular Ca^{2+} dependent anti-coagulant, possesses a high affinity for PS it can be utilized to detect these early events of apoptosis (Andree HAM et al., 1990). For the detection of translocated PS Annexin V is linked to fluorophores such as fluorescein isothiocyanate (FITC). In fact the PS translocation is not unique to apoptosis but can also be observed during necrosis. In order to distinguish between necrosis and apoptosis using the Annexin V system a dye exclusion test is therefore performed additionally. While during the initial stages of apoptosis the cell membrane stays intact it loses its integrity during necrosis (Vermes I et al., 1995; Engeland v. M, 1998). Hence if the dye is exclusively detectable on the cell's exterior the cell can be considered as apoptotic and not necrotic.

Cell Viability and Cytotoxicity Assays Since cell viability, cell vitality and compound cytotoxicity are key characteristics in the drug evaluation a large panel of cell viability and cytotoxicity assays are available from a multitude of companies. The ideal *in vitro* test to assess cell viability and cytotoxicity must be rapid, reliable, safe, sensitive, simple and economical. Furthermore, since these assays are used to

assess drug efficacy of NIDs the assay must not interfere with the candidate drug. (O'Brien J et al., 2000). Generally speaking there are conventional reporter-based cell assays and cell-based label free approaches. Using reporter-based cell assays can generate artifacts due to genetic alterations of the cells or the reporter-protein related disturbances of the target signaling pathways. Since labeling can alter cell physiology and hence sensitivity to certain potential drugs label-free assay might predict drug efficacy more reliable than reporter-based cellular assays (Abbit KB et al., 2000).

One possible means of assessing cell viability in a label-free approach is to determine the ATP content present in the cells. Almost all cellular processes which require energy utilize the potential of ATP as energy source; consequently the amount of ATP in a metabolically active cell correlates with its viability. ATP is exclusively generated during mitochondrial processes. The production of ATP is stopped within minutes after the loss of membrane integrity (i.e. apoptosis and necrosis) and the actual ATP level present in the cell is reduced sharply due to endogenous ATPases. By utilizing bioluminescence the amount of ATP can be quantified very rapidly. The luciferase system (e.g. CellTiter-Glo[®], Promega) requires ATP and luciferin as substrates to generate bioluminescent light directly proportional to the amount of ATP present. An inherent limitation of common luciferase assays is the short half-life of the luminescent emission due to the degradation of ATP by ATPases. This can be overcome by pH dependent inactivation of ATPases or by adding ATPase inhibitors. Additionally candidate drug compounds may also interfere with the firefly luciferase resulting ultimately in false positives. Promega's CellTiter-Glo[®] system therefore utilizes a proprietary Ultra-Glo[™] rLuciferase, which is less sensitive to compound related inhibition (Hook B and Schagat T, 2011).

A second target for the determination of cell viability is the respiratory chain and its underlying oxidative phosphorylation. Since oxidative phosphorylation involves the transport of electrons via several membrane proteins of the mitochondrion many reduction-oxidation (redox) reactions are involved. This fact can be used to monitor

cell viability due to the reducing power of metabolically active cells to convert substances such as alamarBlue® (Life Technologies) to a fluorescent molecule by chemical reduction (O'Brien J et al., 2000). The active ingredient of alamarBlue® is resazurin (Figure 6), a nontoxic, cell permeable bluish compound that is reduced to resorufin in viable cells producing red fluorescence (according to Life Technologies, O'Brien J et al., 2000). This fluorescent signal is direct proportional to the number of living cells and upon excitation at 570 nm the fluorescence peak emission is detected at 585 nm. Additionally, alamarBlue® can also be detected via absorbance at 570 nm which lowers sensitivity of the measurements. Despite its simplicity, some drawbacks to the routine use of alamarBlue® were observed. First the accumulation of resorufin was shown in tests with several toxicants such as 0,3-1mM nickel chloride which ultimately could result in an overestimation of cell number. Second the continuous reduction of resorufin produces hydroresorufin, a colorless and non-fluorescent molecule. This over-reduction which is due to high metabolic rates of certain cell types leads to an underestimation of cell viability and metabolic activity. This ultimately could lead to artifactual results and misinterpretations in drug evaluation (O'Brien J et al., 2000).

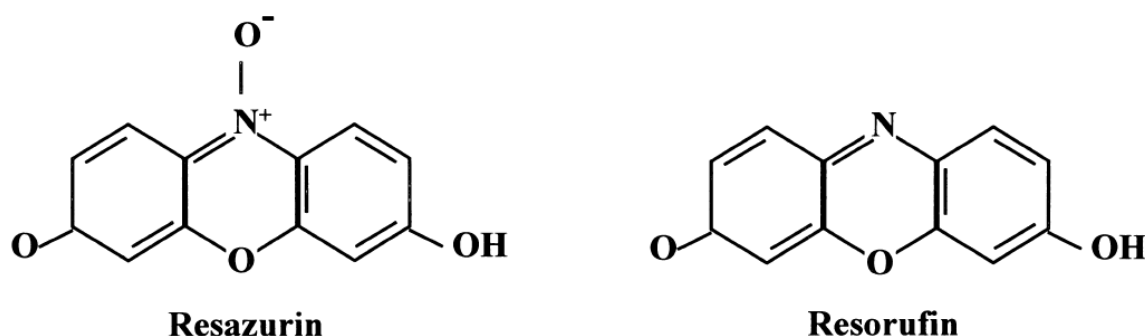


Figure 6: Chemical reduction is the mechanism of alamarBlue® as an indicator for cell viability.

Resazurin, a non-fluorescent molecule is converted by means of the redox potential of the respiratory chain in living cells. The resulting resorufin is a red-fluorescent molecule which can be measured and correlated with viability of a cell population. Modified picture by O'Brien J et al., European journal of biochemistry/FEBS, 2000.

Senescence Assays | Normal somatic cells do not replicate indefinitely but enter cellular or replicative senescence after a defined number of cell divisions. This

process is the underlying cause of aging. Furthermore, this is of prime importance since immortality of cells increases the susceptibility to malignant transformation. Hence the process of senescence is thought to be an important mechanism to avoid tumorigenesis (Dimri GP et al., 1995).

Senescence-associated β -galactosidase (SA- β -Gal) is an enzyme that is expressed in cells originating from all three germ layers. It is probably the most commonly used indicator for senescence. Since SA- β -Gal is expressed only in senescent cells, pre-senescent, quiescent or immortal cells are negative for SA- β -Gal. The enzymatic activity of SA- β -Gal can be utilized to determine senescence. X-gal, a substrate of β -galactosidase, is converted by β -galactosidase to a bluish measurable precipitate. Therefore the presence of β -galactosidase can be assessed using this characteristic. Nevertheless, in order to detect the presence of SA- β -Gal a biological characteristic of SA- β -Gal can be exploited. At pH 6.0 only SA- β -Gal is active while lysosomal β -Gal (expressed in most cells) shows optimal activity at pH 4.0. Therefore, if the senescence assay is performed at pH 6.0 only senescent cells are stained blue (Dimri GP et al., 1995). This finally allows for determining the effect of the candidate drug on senescence.

Hypoxia Assays | As discussed previously in order to assure the supply with sufficient nutrients and oxygen the cells must reside within ~100-200 μ m of vasculature. To maintain the state of sufficient oxygen supply (normoxia) is not only essential for normal but even more for highly proliferative malignant tissue. Regularly a tumor faces a state of diminished tissue oxygen tension during tumorigenesis, a state that is called hypoxia, since tumor growth may even outpace angiogenesis. For this purpose cells have intrinsic ways such as reduction of energy consumption and anaerobic metabolism to withstand the resulting fluctuations in oxygen supply (Simon MC, 2010). Cancer cells could utilize inherent programs to adapt to hypoxic environments and to avoid hypoxia induced cell death. More than that, it was issued that the probability of invasion and metastasis are positively correlated with the degree of intratumoral hypoxia since undersupply with oxygen and nutrients may

force cancer cells to get access to distant vasculature (Zhong H et al., 1999). Many genes are differentially regulated during these programs that finally govern angiogenesis, metabolism and cell survival. Hypoxia inducible factors 1,2 (HIF-1 and HIF-2) are transcription factors that play a central role in these phenotypic shifts of the cells (Simon MC, 2010). It was shown that HIF-1 α is highly overexpressed in a broad range of cancers upon exposure to hypoxic conditions (Zhong H et al., 1999). Giving an example the expression of vascular endothelial growth factor (VEGF) and the glucose transporter 3 (GLUT-3) is up-regulated by HIFs. The angiogenic VEGF is of prime importance in the formation of blood vessels *de novo* (neovascularization). Blood and therefore oxygen supply might be assured within the malignant tissue by up-regulating the expression of VEGF. Nevertheless, it was reported that the vasculature formed by this tumor-induced process is often inadequate due to its rather leaky and defective structure and may not supply the tumor with nutrients. As a consequence even vascularized tumors could comprise hypoxic areas in which the HIF induced programs are up-regulated. Moreover, due to the increased expression of GLUT-3 the glucose influx into the cells is enhanced. This results in the production of sufficient amounts of ATP by the anaerobic glycolysis. The ability to induce angiogenesis and the HIF-induced programs are potential targets for anti-cancer drugs. Common assays to determine HIF-1 presence in cell lysates are sandwich ELISAs such as the HIF1 α EZ-TFA Transcription Factor Assay (EMD Millipore) which enables chemiluminescent detection.

Proteome Analysis using Post-Translational Modification Assays| After transcription and translation the majority of proteins do not have their final structure after they emerge from the ribosomal biosynthesis machinery. It is known that a set of more than 30 different modifications can alter amino acid residues of proteins by proteolytic cleavage or addition of a modifying group. These post-translational modifications (PTMs) are essential since they may determine the protein's activity, state, localization, turnover, and interactions with other proteins. Consequently, PTMs are essential to regulate signaling pathways of which many of those are in key

positions of regulating certain aspects of cell behavior such as proliferation and cell survival (Alberts B et al., 2008; Mann M and Jensen ON, 2003).

Phosphorylation represents a frequent PTM and plays among others a central role in tumorigenesis. In our genetic repertoire hundreds of protein kinases and phosphatases are known to modify proteins on serine, threonine, or tyrosine amino acid side chains thereby generating a multitude of protein molecular variants (Walsh C, 2006). This reversible modification represents a molecular switch for the activation/inactivation of enzyme activity, modulation of molecular interactions and signaling, which may ultimately regulate proliferation, cell survival and tumorigenesis (Mann M and Jensen ON, 2003). For this purpose it is essential to determine the phosphorylation patterns of certain protein panels known to be involved in tumorigenesis. This would allow unraveling the complexity of tumor-associated signaling and its underlying mechanisms cancer cells could exploit to evade anti-cancer treatments and ultimately to identify new anti-cancer drug targets.

A convenient method to detect changes in phosphorylation of multiple crucial proteins at once is the Proteome Profiler Antibody Array™ (R&D Systems®, Abingdon, Oxon, UK). The underlying protein array technology allows for screening of more than 40 different phosphorylated proteins in a micro-array like approach. This profiling is enabled by highly sensitive antibodies which are directed against specific phosphorylation patterns of certain target proteins. The underlying methodology is similar to a sandwiched western blot where the capture antibodies are spotted on a nitrocellulose membrane. After binding the respective protein (in a lysate sample) a detection antibody conjugated with horseradish peroxidase (HRP) sensitive to phosphoproteins binds to the complex. Bioluminescence-based readout allows for highly sensitive detection of changes in phosphorylation patterns of the investigated samples (R&D Systems, 2011).

2.4 Engineering of Pathological Tissues for Anti-Cancer Drug Discovery

In vivo, cells and extracellular matrix (ECM) form tissues, which join together in the structural and functional units of organs that communicate with each other and are interlinked by vasculature to ultimately form a complex organism (Egeblad M, 2010). Consequently, the organ's function can be traced back to the activity of specialized cells in a complex network. The stroma consists of extracellular macromolecules (proteoglycans, hyaluronic acid, fibronectin, collagen and laminin) constituting the extracellular matrix (ECM) and various types of stromal cells. The stromal cells include fibroblasts, adipocytes, cells of the vascular and the immune system. It was once thought that the ECM serves primarily as a framework which stabilizes the tissues. Today it is commonly accepted that this scaffold even can regulate cell morphology, differentiation, proliferation and cell survival to outline just a few (Alberts B et al., 2002). Even though many advances have been produced by standard two-dimensional cell culture studies data from more physiological 3D cultivation approaches show the critical impact of three-dimensionality on cells and hence evaluation of drug efficacy. Due to the properties of the ECM to determine a cell's fate it seems apparent that the specific cellular and matrix microenvironment provided to cells would have a crucial impact on the sensitivity of cells towards drug application. Hence 3D cultures are currently being exploited in many studies focusing on invasion/metastasis, angiogenesis, tissue modeling and remodeling, and might be highly predictive models in drug discovery in the near future.

2.4.1 Commonly Used In Vitro 3D Models

Basically there are two different approaches to establish *in vitro* 3D organotypic models for a multitude of different studies. In the first approach whole tissue samples are harvested *in vivo* for subsequent cultivation *in vitro*. Second the 3D models are established using isolated cells from already established cell lines or dissociated tissues (Yamada KM and Cukierman E, 2007).

Ex vivo tissue explants as *in vitro* models | Since tissues have a unique and highly complex 3D architecture *ex vivo* tissue explants serve as a promising source for 3D *in vitro* models to study diseases and their underlying molecular mechanisms. Since these explants comprise a heterologous context of several different cell types 3D *in vitro* co-culture models can be used to reveal unknown cell-cell and cell-ECM interactions, and their related control mechanisms that regulate cellular differentiation, proliferation and apoptosis (Kim JB, 2005). Since these are key characteristics in evaluating pharmacological properties of novel anti-cancer *drugs* *these* models are promising tools in modern drug discovery. Hypoxia and nutrient insufficiency are reported to be present in most tumors *in vivo* with a size beyond several mm³ which ultimately could result in necrosis (Dang VC and Semenza GL, 1999). Normal and transformed cells utilize inherent programs to avoid hypoxia induced cell death and to adapt to these hostile environments. Hence tumor biology could benefit from these 3D tissue models to study the induction of angiogenesis and resistance to anti-cancer drugs *ex vivo* (Hicks KO et al., 2006). As discussed previously vasculature is essential for tissue maintenance since sufficient oxygen and nutrient supply can only be ensured for cells residing within ~100-200 µm around blood vessels. Only recently, promising approaches were reported to generate blood vessels *in vitro* using embryonic stem cells (ESCs) (Kreuger J et al., 2006; Jakobsson L et al., 2007). Nevertheless, generating functional vasculature in *in vitro* 3D tumor models is more than a challenging mission which definitely will be a focus in future tissue modeling studies. In order to maintain the tissues under normoxic conditions the harvested viable tissues are sliced in ~0.1-0.3 mm thin slices prior to further cultivation. This approach is facilitated by using specialized devices such as microtomes (e.g. Krumdieck, Alabama Research and Development Corp., Munsford, USA), or more recently vibratomes (e.g. Leica VT1200 S, Leica Microsystems, Wetzlar, Germany). These instruments allow for gentle treatment of the samples to ensure the generation of highly viable tissue slices (van der Kuip H et al., 2006; Zimmermann M et al., 2009). Subsequently the thin slices can be cultivated then either on organotypic supports (such as Teflon membranes with 0.4-

µm pores, Millipore) and submerged in serum-supplemented specific media or are embedded into ECM scaffolds (Vaira V et al., 2010).

Despite its potential the inherent limitation of this approach is its transitory nature which allows basically only relatively short term culture. In addition, to ensure viability of tissue slices the harvested tissues (residual tissue not needed for diagnostic purposes) must be transported from the operating rooms to the laboratory within the shortest time period possible (~30 min, Gramignoli R, 2011). The collection of human tissues for research and drug discovery studies must follow ethical and institutional guidelines (informed consent).

In addition, predicting the response of an individual patient to a certain anti-cancer therapy, i.e. personalized medicine, is of paramount importance. *Ex vivo* tissue explants might be a promising approach to evaluate the pharmacological properties of anti-cancer drugs related to the individual cancer patient by using in high-content phenotype based drug discovery (Vaira V et al., 2010).

***In vitro* models established from isolated cells** In a second approach to establish *in vitro* 3D organotypic models isolated cells are used instead of whole tissue samples. These cells originate either from cell lines, dissociated primary tissues or stem cells. After the isolation the cells are propagated *in vitro* prior to embedding as single cells or cell aggregates in natural (Matrigel™, fibronectin, collagen, laminin) or synthetic (Extracell™, self-assembling peptide nanofibers) ECM scaffolds. Since in these models the cells attach to surrounding macromolecules of the ECM rather than to two-dimensional coated surfaces, matrix composition and rigidity have crucial influence on cell signaling and cell behavior (Cukierman E et al., 2002). As a logical consequence the drug response of cells cultured in three dimensions might be completely different compared to cells cultured on conventional two-dimensional coated surfaces.

Another important aspect of physiological niches is the presence of stromal cells which is known to be a crucial feature in the early stages of cancer metastasis. For anti-cancer drug discovery studies it is therefore essential to provide the tumor with stromal cells in order to evaluate the drug's pharmacological properties more

reliable. For this, due to the ability of malignant cells to form spontaneously coherent cell-aggregates with other cells (heterotypic aggregation), heterotypic co-cultures can be reproducibly established (Kim JB, 2005). These individually designed heterotypic co-cultures can comprise virtually all stromal cell types which allow evaluating the drug's pharmacological properties more reliable. Since cellular interactions and activation (e.g. gene expression, cellular crosstalk, cell behavior and proliferation) is controlled by the local surrounding the microenvironment is determining the cell's fate. Nevertheless it is not only the neighborhood of cells which is crucial but also the defined composition of extracellular macromolecules (e.g. fibronectin, collagen and laminin) and mediators (e.g. hormones, cytokines and growth factors) present. Only fine-tuned physico-chemical and biological parameters ensure optimal micro-environmental conditions that mimic the *in vivo* situation as closely as possible (Figure 7).

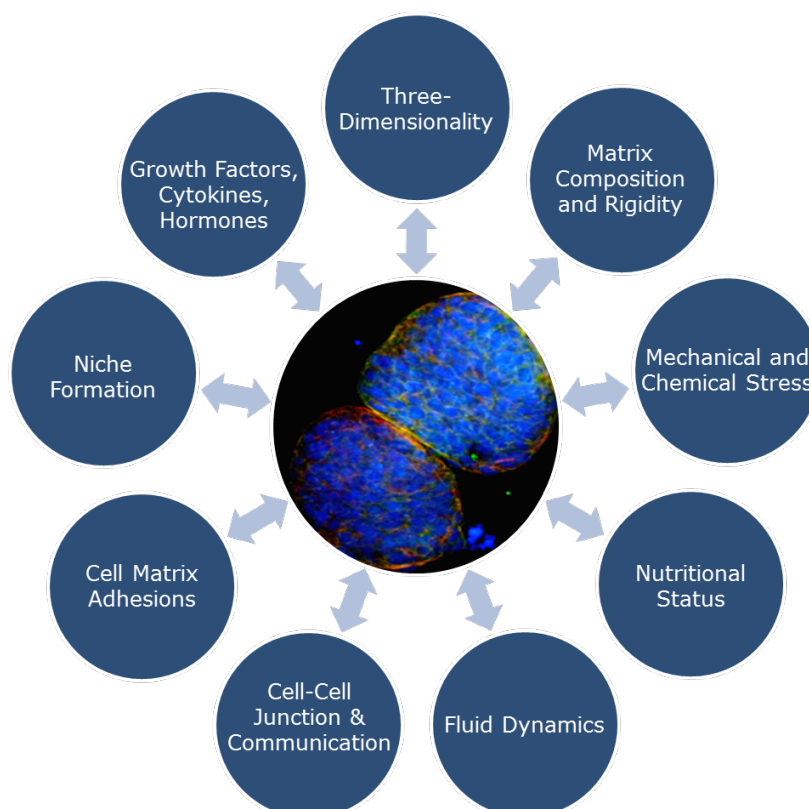


Figure 7: Environmental factors that affect cell behavior and consequently the evaluation of drug efficacy.

Many aspects must be taken into consideration when generating organotypic microenvironments. The cells or cell aggregates (spheroids, center) are heavily dependent on various parameters which must be fine tuned in order to ensure an accurate reflection of cell behavior *in vivo*.

Every tissue *in vivo* possesses certain characteristics which are defined by their molecular compositions. Dependent on the cancer type each 3D tumor model therefore must comprise appropriate molecular and macroscopic architecture in order to mimic the phenotype of different tissues appropriately *in vitro*. Natural biomaterials including purified ECM proteins such as collagen or fibronectin and ECM derivatives such as Matrigel™ or SIS™ are commonly used in tissue engineering. Due to their natural origin these biomaterials can mimic function and structure of the native extracellular environment very closely. Beside natural biomaterials also a broad range of synthetic polymers are being used as scaffolds for 3D models, i.e. poly (α -hydroxy acids) or aliphatic polyesters such as PLA, PGA and PLGA (Marx U and Sandig V, 2007). The ability to control rigidity, processability, inertness, microstructure and permeability very precisely is one of the main advantages of synthetic materials (Vacanti JP and Langer R, 1999).

In addition, the different cell types in this heterotypic tumor-model can be stably and differently labeled with fluorescent proteins using e.g. retroviral vectors. This allows for tracking the fates of the different cell types and studying the effect of cellular crosstalk on drug efficacy.

The combination of individual tumor cell-aggregates, various biological or synthetic ECM scaffolds and stromal cells of any lineage makes this approach so versatile and powerful for future anti-cancer drug discovery.

2.5 Objectives of this Master Thesis

Today's classical approach to evaluate new anti-cancer drugs is to screen large libraries of chemically synthesized compounds on two-dimensionally cultured cancer cell lines. Since life is three-dimensional it will apparently lead to the conclusion that three-dimensional cues might be essential in the regulation of cell physiology. Two-dimensionally grown cells are not in their ecological niche by which they are regulated by a multitude of stimuli. It is well known that 3D cultures can maintain biochemical and morphological features similar to those found in their corresponding tissues *in vivo* – a fact, that makes them superior over conventional monolayer or suspension cultures. Nevertheless, it still remains elusive if 3D models could fill the gap between conventionally cultured cancer cell lines and clinical studies in the drug developmental process.

Since lung cancer accounts for more deaths than any other cancer type we focus on the most frequent (80%) lung cancer subtype, Non-small cell lung cancer. The aim of this thesis is to develop an organotypic three-dimensional lung cancer model as an HTS-amenable test system that mimics the human tissue microenvironment as accurate as possible. For this purpose, different methods to generate multicellular spheroids need to be evaluated for their applicability or must be newly developed.

Different methods to generate spheroids:

- Hanging Drop
- Rotary Cell Culture System (RCCS)
- Methylcellulose; formation of spheroids in semisolid media
- Coated surfaces that inhibit cell attachment (poly-HEMA, HydroCell™)

Each tissue has its unique composition and therefore individual physico-chemical properties. To mimic the ideal microenvironment for lung cancer cells we evaluate different matrices for their applicability in that model.

Natural vs. synthetic ECM-surrogates:

- Natural Source:
 - Matrigel™
 - Rat tail collagen type I
- Synthetic Material:
 - HyStem; Hyaluronan-based hydrogel
 - HydroMatrix™ peptide nanofibers scaffold

To compare expression and localization of marker proteins in 2D and 3D, protocols for immunofluorescent staining of embedded spheroids need to be developed.

In order to determine the impact of cellular crosstalk on the invasive potential of tumor cells heterotypic co-cultures of tumor and stromal cells must be established.

One possibility to discriminate between different cell types in co-cultures is by transduction of the cells to stably express fluorescent proteins. For this the lentiviral transduction system LentiORF™ (Thermo Fisher Scientific), will be used to transduce human fibroblasts and lung cancer cells.

Microarray analyses and Phospho-Proteome Antibody Arrays will be used to determine differential gene expression and altered phosphorylation patterns of cancer-related proteins.

The therapeutic efficacy of a candidate anti-cancer agent can be evaluated by means of its ability to induce the reversion of the acquired cancer hallmark capabilities. We chose two conventional chemotherapeutics (paclitaxel, Cis-Diamminedichloroplatinum) and a SMWC targeting the EGFR (Gefitinib). It has been consistently reported that a majority of lung cancer patients carry EGFR mutations which strongly influence the progress of disease.

3 Material and Methods

All described procedures in this section were conducted aseptically unless otherwise stated.

3.1 Cell Culture

Primary neonate human dermal fibroblasts (nHDF), obtained from Innoprot (Derio, Spain), were cultivated in fibroblast growth medium (Promocell; C-23010). The lung adenocarcinoma cell lines HCC827, NCI-H1437 and NCI-H1975 were obtained from the American Type Culture Collection (ATCC; Rockville, Maryland) and cultivated in RPMI-1640 (Gibco, Life technologies) growth medium supplemented with L-Glutamine, Penicillin/Streptomycin and fetal bovine serum (HCC827, 15%; NCI-H1437 and NCI-H1975 10%). The lung epidermoid carcinoma cell line CaLu-1 was obtained from the American Type Culture Collection (ATCC; Rockville, Maryland) and cultivated in McCoy's 5A-GlutaMAX growth medium (Gibco, Life technologies) supplemented with L-Glutamine, Penicillin/Streptomycin and 10% fetal bovine serum. All cell lines were propagated using 0.25% Trypsin-EDTA. Trypsin was inactivated by the addition of fresh growth medium and cells were harvested by centrifugation at 240 x g for 3 min. Neonate HDF cells were centrifuged for 5 min at 240 x g. Cells were grown in T25, T80 and T175 cell culture flasks and 100 mm petri dishes w/Nunclon™ Δ surface (Thermo Fisher Scientific, Huntsville, AL, USA).

3.2 Spheroid Generation

In order to produce spherical cell aggregates termed spheroids, conditions in which the adhesive forces between the cells are greater than for the substrate it is plated on are required (Kim JB, 2005). For this various methods were used.

3.2.1 Hanging Drop Method

The cells were suspended in 20 μL droplets of medium where they spontaneously develop into coherent spherical cell aggregates within 1-2 days of incubation at 37°C and 5% CO_2 in a humidified atmosphere.

Starting from a cell monolayer the cells were harvested as described above (see *Cell Culture*), counted and the concentration was adjusted to 2500 cells/ mL. 20 μL of this cell suspension was dispensed onto the inward of an inverted 100 mm culture dish lid. The lid was inverted and placed on the dish bottom containing 10 mL of 1x Phosphate buffered saline, PBS, (Life Technologies, Cat#10010-023) to avoid evaporation of droplets during incubation (Figure 8). After 48h of incubation at 37°C and 5% CO_2 in a humidified atmosphere the spheroids were harvested by inverting the lid and aspirating the droplets to collect the suspension in a conical tube. Since the spheroids within a certain cell type are of similar shape and diameter (e.g. HCC827 $\sim 550\ \mu\text{m}$; NCI-H1975 $\sim 800\ \mu\text{m}$) the sedimentation time was determined and the clear supernatant could be aspirated after 5 min in order to adjust the spheroid concentration for further experiments.

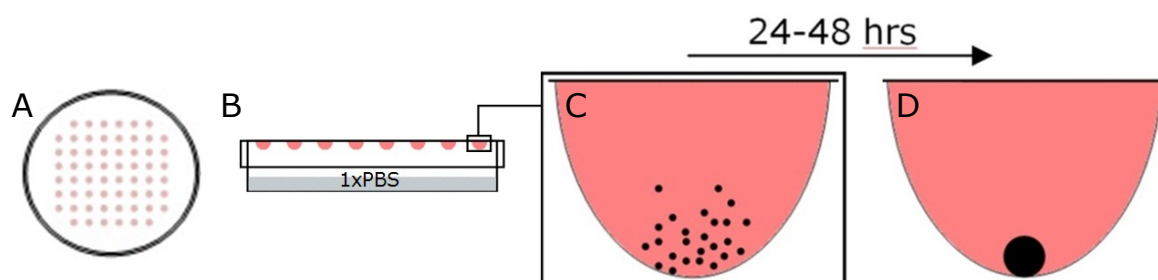


Figure 8: Formation of multicellular tumor spheroids by hanging drop method.

A] 20 μL droplets of cancer cell suspension (2500 cells per mL) are dispensed onto 100 mm culture dish lid. **B]** The lid is inverted and placed on the culture dish tray with a reservoir of 1x PBS to avoid evaporation of medium during incubation. **C]** Within 24-48h the cells sink to the bottom of the droplet and form cell-cell contacts. **D]** The resulting multicellular spheroid (one per droplet) is supplied with sufficient nutrients and growth factors by the growth medium while residing on the bottom of the droplet. After formation the spheroid is harvested for further experiments.

3.2.2 Spheroid Generation via Rotary Cell Culture Systems™

By simulating microgravity the by NASA developed rotary cell culture system (RCCS) maintains cells in a suspension with low hydrodynamic forces which allows the cells to aggregate and to form for spheroids (Figure 9). Due to a semi-permeable

membrane each vessel provides sufficient aeration and inhibits bubble formation which otherwise would result in high shear forces (Kim JB, 2005).

After equilibrating the 10 mL rotary wall bioreactor vessel (RCCS-4DQ, Synthecon Inc., Houston, Texas) with 1xPBS for at least 30 min, the cells were harvested and counted as described above (see *Cell Culture*), 1×10^6 cells were seeded into each vessel and incubated at 37°C and 5% CO₂ in a humidified atmosphere. For each specific cell line optimal incubation time and rotation speed were determined (e.g. HCC827 48-72h at 6.3 rpm; NCI-H1975 48h 7.0 rpm). To ensure optimal spheroid formation the individual growth medium was replaced after 48h. After cell line dependent duration of incubation the spheroids were harvested, counted and the desired concentration for further experiments was adjusted after centrifuging the spheroid suspension for 10 s at 235 x g and aspirating the respective excess volume of supernatant.



Picture by EXMED3d

Figure 9: Rotary Cell Culture System (RCCS)

The NASA developed RCCS allows for the generation of 3D cell aggregates due to low shear force, high mass transfer and microgravity. The 4x2 station rotation base allows the installation of 8 RCCS bioreactor vessels. Via 4 tachometers on the power supply unit the individual rotation speed can be adjusted for pairs of two vessels respectively.

3.2.3 Spheroid generation on coated surfaces to inhibit cell attachment

To induce spontaneous spheroid formation, conditions in which the adhesive forces between the cells must be greater than for the substrate it is plated on. For this purpose cells are grown either in semisolid medium (Methylcellulose), on semisolid

substrate (Agarose) or on substrates coated with plastic which prevent cell attachment (poly-HEMA, NUNC Hydrocell plates).

Methylcellulose solution In order to prepare the highly viscous methylcellulose solution 1.2 g of the pure powder (Sigma Aldrich, Cat#M0512, 4000 Centipoises) were autoclaved. Subsequently the powder was dissolved in 50 mL pre-warmed basal medium (60°C) for 20 min under continuous stirring. After the addition of 50 mL basal medium (RT) the solution was mixed by rotation overnight (o/n) at 4°C. This solution was centrifuged at 5,000 x g for 2h at RT. The highly viscous clear supernatant, comprising 90-95% of the total volume, was finally used for spheroid generation upon 5-fold dilution with the cell suspension of desired concentration. For this purpose 200 µL of the cell suspension comprising 40 µL of methylcellulose solution was dispensed per well of a round bottom 96-well suspension culture plate (Greiner, Cat#650185) and subsequently centrifuged at 850 x g for 2 min. Cells were incubated at 37°C and 5% CO₂ in a humidified atmosphere to develop spontaneously into spheroids within 2 days.

Agarose coating For coating of various surfaces to inhibit cell attachment and to promote spontaneous spheroid formation 1% agarose solution dissolved in growth medium was used. For this purpose 1 g of agarose was dissolved in 100 mL sterile basal medium by repeated heating using a microwave oven. Immediately after complete dissolution 500-750 µg agarose were added per cm² surface area. Subsequently, the hot agarose solution was aspirated and incubated at 37°C for 30 min. The cell suspension intended to form spheroids was added to the residual agarose film and incubated for 24-48 hours at 37°C and 5% CO₂ in a humidified atmosphere.

Poly-HEMA coating To prepare a 1.2% poly 2-hydroxyethyl methacrylate (poly-HEMA) solution for generating surfaces which prevent cell attachment 2.4 g poly-HEMA (Sigma Aldrich, Cat#P3932) was dissolved in 20 mL 95% ethanol for 8h at 65°C under continuous stirring. Subsequently, this solution was 10-fold diluted with

95% ethanol to a final poly-HEMA concentration of 1.2%. Surfaces were coated using 0.8 – 1 mg/cm² final coating concentration. The solution was dried at 37°C o/n in the oven. After evaporation, the cell suspension was added in the desired concentration (Table 1) onto the poly-HEMA coated surface and incubated for 24-48h at 37°C and 5% CO₂ in a humidified atmosphere to form spheroids.

Generation of Spheroids in poly-HEMA coated flat bottom 96-well plate		
Cell Line	Number of Cells/well [10 ⁴]	Incubation Period [hours]
CaLu-1	1.5	18
HCC827	1.5	48
NCI-H1437	2	36
NCI-H1975	1	24

Table 1: The table indicates the optimal cell number for the generation of spheroids of each of the tested lung cancer cell lines in poly-HEMA coated flat bottom 96-well microtiter plates.

3.3 Embedding of Spheroids

Spheroids were embedded in matrices consisting of Matrigel™ (Basement Membrane Matrix, BD Biosciences, Cat#354234) collagen type 1 (Rat Tail Collagen, BD Biosciences, Cat#354236) or mixtures of these.

Matrigel was removed from frozen storage (-20°C) and placed on ice to thaw o/n at 4°C until ready for use. BD Biosciences' formulation of rat tail collagen type 1 in 0.02N Acetic Acid started to gel when its pH was brought to alkalinity. Upon addition of 1N NaOH (2.3% of the final volume of collagen to be used) and 10xPBS (10% of the final total volume; pH 7.4, Life Technologies, Cat#70011-069) the collagen formulation was close to physiological pH and started to gel as indicated through advancing turbidity. The spheroid suspension (see *Spheroid Generation*) was used to dilute the matrix (Matrigel, collagen type 1) to the desired final concentration. The spheroid-matrix suspension was dispensed into the appropriate labware (see [Table 2](#)). Complete gelation of matrix was achieved by incubating at 37°C for approximately 60-180 min dependent on matrix volume and labware. After entire

gelation the matrix was overlaid with appropriate medium for subsequent incubation at 37°C and 5% CO₂ in a humidified atmosphere.

Matrix Volumina in different Labware

Labware	Surface Area [cm ²]	Matrix Volume [μL]
96-w fl btm	0.33	60-100
48-w	0.95	90-200
24-w	1.91	150-400
8-w Lab-Tek™	0.8	100-150

Table 2: The table indicates the working ranges of matrix volumes used in the different lab wares. In addition the specific surface areas of each used lab ware are listed. w, well; fl btm, flat bottom.

3.4 Immunostaining

Immunostaining of lung cancer cell lines for the characterization of marker protein expression was performed in 8-well Lab-Tek™ labware (Thermo Scientific, Cat#177445) with the Permanox® resin slide. Immunostaining was performed under non-sterile conditions.

3.4.1 Two-dimensional monolayer

48h after cell seeding the wells were washed with prewarmed (37°C) Hanks' Balanced Salt solution (HBSS, Life technologies, Cat#14025). After 30 min of fixation with 2.5% formalin (Carl Roth, Cat#4980.1) in HBSS the cells were washed with HBSS following PBS. Cells were permeabilized with 0.2% Triton® X-100 (Sigma Aldrich, Cat#T8787) in PBS for 15 min, washed with PBS and subsequently treated for 20 min with 50 mM NH₄Cl/0.1% Glycine in PBS (quenching of background prior to antibody incubation). Incubation of 1st antibody (Table 3) was performed o/n at RT with consequent 1xPBS washing. Incubation of 2nd antibody (Table 3) was performed for 1 hr at RT following wash with 1xPBS and deionized water. Additionally fluorescent-phalloidin (Alexa-Fluor® 568-phalloidin, Life Technologies, Cat#A12380) was diluted 1:800 in 2nd antibody solution to stain actin filaments. After removal of chambers and gaskets, VECTASHIELD® mounting medium containing

DAPI (Vector Laboratories, Cat#H-1500) was added to the coverslips and dried o/n at RT protected from light.

3.4.2 Three-dimensional organoids

Spheroids were embedded in matrices consisting of Matrigel™ (Basement Membrane Matrix, BD Biosciences, Cat#354234) and collagen type 1 (Rat Tail Collagen, BD Biosciences, Cat#354236). Immunostaining of embedded microtumors for the characterization of marker protein expression was performed as described above for two-dimensional Immunostaining with a few adaptations. After the spheroid generation the cell aggregates were embedded in ECM (50 µL Matrigel™, 50 µL Collagen I, 50 µL growth medium). Samples were incubated with regular medium exchange for 5-8 days at 37°C and 5% CO₂ in a humidified atmosphere prior to immunostaining. Organoids were fixed for 1 hr using 3% formalin solution. Permeabilization and quenching was prolonged to 25 min. Secondary antibody incubation was prolonged to 3-4h.

Table A Primary Antibodies used in Immunostaining

Antibody	Antigen (Epitope)	Dilution (in 1xPBS)	Supplier / Product No
Mouse mAb	β-catenin	1:100	BD Biosciences/#610154
Mouse mAb	E-cadherin	1:100	BD Biosciences/#610182
Mouse mAb	Integrin beta 1	1:100	Abcam/#ab3167
Mouse mAb	Ki67	1:100	Dako/#M7240
Goat pAb	N-cadherin	1:100	Santa Cruz/#SC-1502
Mouse mAb	pan Cytokeratin	1:100	Abcam/#ab17155-1
Mouse mAb	Vimentin	1:100	Dako/#M0725
Rabbit pAb	ZO-1	1:100	Abcam/#ab59720-50

Table B Secondary Antibody Fragment Conjugates used in Immunostaining

F(ab') ₂ -Conjugate/Host	Antigen (Epitope)	Dilution (in 1xPBS)	Supplier / Product No
Alexa Fluor 488/Goat	anti-mouse	1:400	Life Technologies/#A11017
Alexa Fluor 488/Goat	anti-rabbit	1:400	Life Technologies/#A11070

Table 3: The tables list the primary antibodies (A) and secondary antibody fragments (B) used in immunostaining experiments. Mouse, Rabbit, Goat: the species of the host animal in which the antibody was raised; mAb: monoclonal antibody; pAb: polyclonal antibody.

3.5 RNA Preparation

Even though gene expression studies have focused mainly on mRNA levels smaller RNA species like miRNA take center stage in the last years. In order to purify total RNA including miRNA from 2D and 3D cultures Qiagen's miRNeasy Mini Kit (Qiagen, Cat#217004) was used.

3.5.1 Two-dimensional cell monolayer

48-72h before RNA preparation 1.5×10^6 cells were seeded per 100 mm culture dish (Nunc™, Cat#150350). To prepare total RNA from cell monolayer the cells were washed on the day of RNA preparation with 1xPBS before QIAzol® lysis reagent was added. After 5 min of incubation at RT the cell lysate was transferred into centrifuge tubes and chloroform was added (1/5 of the total QIAzol® volume added). The tubes were placed at RT for 3 min for subsequent centrifugation at 12,000 x g for 15 min at 4°C. The resulting upper aqueous phase (70-80% of total volume) was transferred and 1.5 volumes of 100% ethanol were added and mixed. After several centrifugation steps (according to manufacturer's instructions) using the RNeasy Mini columns, 1.5 µL of the samples containing the purified RNAs was used for RNA quality determination (see *Experion™ RNA Quality Determination*); the remaining samples were stored below -80°C for RNA expression profiling using affymetrix gene chip technology.

3.5.2 Three-dimensional organoids

72-96h after seeding single cells into the rotary cell culture bioreactor (RCCS-4DQ, Synthecon Inc., Houston, Texas) spheroids were harvested, embedded in Matrigel/collagen type I matrices diluted with growth medium (equal amounts) and incubated in 48-well plates (Greiner, Cat#677102) at 37°C and 5% CO₂ in a humidified atmosphere. 6 days after embedding, matrices were washed with 1xPBS before QIAzol® lysis reagent was added and protocol conducted as described previously in RNA Preparation of two-dimensional cell monolayer (see previously).

3.5.3 Experion RNA Quality Determination

Preliminary RNA quality tests before RNA expression profiling were performed using the capillary gel electrophoresis platform Experion™ and Experion RNA StdSens Analysis Kit (Cat#700-7104) by Bio-Rad. For quality determination the 1.5 µL of RNA samples prepared using the miRNeasy Mini Kit (Qiagen) and RNA ladder were heated up for 2 min at 70°C to be immediately cooled down on ice for 5 min. After priming the analysis chips with gel-stain solution using the Experion Priming Station (Cat#700-730) 7.5 µL loading buffer was added to the pre-heated sample, mixed and 6 µL of final mixture was loaded into each well of primed Bio-Rad's StdSens Analysis Chips. After loading 9 µL gel-stain solution and 9 µL unstained gel into the indicated wells the RNA quality was measured on the Experion™ gel electrophoresis platform (Software Version 3.1).

3.6 Determining drug efficacy in lung cancer cell lines by alamar-Blue assay

AlamarBlue® Cell Viability Reagent (Life Technologies, Cat#DAL110) was used for rapid and quantitative determination of cell proliferation and viability.

3.6.1 Sensitivity Testing

Cells grown in Monolayer Serial dilution of lung cancer cell lines CaLu-1, HCC827, NCI-H1437 and NCI-H1975 (ATCC; Rockville, Maryland) ranging from 1×10^5 – 195 cells per well was performed to evaluate the sensitivity of alamarBlue® reagent. 200 µL cell suspension was dispensed per well of a black, clear-bottom 96-well plate (Nunc™, Cat#165305) and incubated for 24h at 37°C and 5% CO₂ in a humidified atmosphere. 20 µL of alamarBlue® reagent was added to each well and incubated for 6h at 37°C and 5% CO₂ in a humidified atmosphere. Data were assessed using the PARADIGM™ Detection Platform (Beckman Coulter/Molecular Devices, Multimode Analysis Software Version 3.3.0.9) and appropriate fluorescence intensity detection cartridge (Beckman Coulter/Molecular Devices, Cat#A56150) at 595 nm.

Spheroids | Additionally serial dilutions of spheroids generated from the same lung cancer cell lines as described above were performed to evaluate the sensitivity of alamarBlue® reagent with spherical cell aggregates. Spheroids were generated using poly-HEMA coated 96-well plates (see *poly-HEMA coating*) and serially diluted ranging from 160 – 5 spheroids in per well of a black, clear-bottom 96-well plate (Nunc™, Cat#165305). After 4 hours of incubation at 37°C and 5% CO₂ in a humidified atmosphere alamarBlue® reagent was added and assay performed as described in the *sensitivity testing of cells grown in monolayer* above.

3.6.2 Viability Assay after Therapeutic or Targeted Therapies

Treatment of cells grown in monolayer | Lung cancer cells were seeded (CaLu-1, 1.5×10^4 ; HCC827, 1.5×10^4 ; NCI-H1437, 2×10^4 ; NCI-H1975, 1×10^4 ; cells per well) into transparent flat bottom 96-well plates (Nunc™, Cat#125174) and incubated for 24h at 37°C and 5% CO₂ in a humidified atmosphere. Cells were treated with Cis-Diamminedichloroplatinum (Sigma Aldrich, Cat#P4394), paclitaxel (LC Laboratories, Cat#P-9600) or Gefitinib (LC Laboratories, Cat#G-4408) and incubated for 96h at 37°C and 5% CO₂ in a humidified atmosphere. Data were assessed (Figures 28, 30) using the PARADIGM™ Detection Platform (Beckman Coulter/Molecular Devices) and appropriate fluorescence intensity detection cartridge (Beckman Coulter/Molecular Devices, Cat#A56150) at 595 nm.

Treatment of spheroids | Spheroids were generated using poly-HEMA coated 96-well plates (see *poly-HEMA coating*) and treated with the four therapeutics depicted for the treatment of cells grown in monolayer mentioned in the previous point.

Treatment of organoids | Spheroids were generated using poly-HEMA coated 96-well plates (see *poly-HEMA coating*), embedded in Matrigel/Collagen Type 1 matrices (see *Embedding of Spheroids*) and incubated at 37°C and 5% CO₂ in a humidified atmosphere. Medium was exchanged after 48h. 4 days after embedding organoids were treated the four therapeutics depicted for the treatment of cells grown in monolayer mentioned above.

3.7 Phospho-Profiling

In order to detect changes in phosphorylation between samples of tumor cells cultured as a monolayer or as three-dimensional organoids various Proteome Profiler™ Antibody Arrays (R&D Systems, ARY001, ARY002B, ARY003 and ARY009) were used. As a representing array the protocol of the human phospho-RTK Array Kit (R&D Systems, Cat#ARY001) is exemplified.

3.7.1 Sample Preparation

48-72h before Phospho-profiling cells were plated at different cell line specific concentrations ($1.5 - 4 \times 10^6$) onto 100 mm culture dishes (Nunc™, Cat#150350). For three-dimensional organoids, spheroids were generated using poly-HEMA coated 96-well plates (see *poly-HEMA coating*), embedded in Matrigel/Collagen Type 1 matrices (see *Embedding of Spheroids*) and incubated at 37°C and 5% CO₂ in a humidified atmosphere in 48-well plates (Greiner, Cat#677102). Medium was exchanged after 48h. 6 days after embedding organoids were lysed. To prepare cell lysates from cell monolayer and organoids the cells were washed with 1xPBS (Life Technologies, Cat#10010-023) before NP-40 lysis buffer (Table 4) was added. For the adequate inhibition of proteases protease inhibitor tablets complete, ULTRA, Mini (Roche, Cat#05892970001) were added supplementary to the lysis buffer. Cells were incubated with lysis buffer for 60 min at 2-8°C on a rocking table. During cell lysis the nitrocellulose membranes were blocked. A small aliquot of the lysates was diluted at a 1:1 ratio with standard Laemmli Buffer containing Dithiothreitol, SDS and Tris-Cl and frozen at -20°C for prospective SDS-PAGE experiments. The lysates were added to the membranes and incubated o/n at 2-8°C on a rocking table. The membranes were washed and the detection antibody (Anti-Phospho-Tyrosine-HRP antibody) was added to each membrane for 2h at RT on a rocking table. Afterwards the membranes were washed, and chemiluminescent western blotting substrate (Pierce, Cat#32106) was added to the membranes. The signal was detected by the

ChemiDoc XRS platform (Bio-Rad, Cat#170-8070) using the high sensitivity mode and analyzed with Quantity One Software.

NP-40 Lysis Buffer		
Components	Proportion/Concentration	Product Information
NP-40	1%	Igepal, Sigma Aldrich; #CA-630
Tris-HCl	20 mM, pH 8.0	Life Technologies; #15568-025
EDTA	2 mM	Life Technologies; #15575-020
NaCl	137 mM	Merck; #1.06404.1000
Glycerol	10%	Merck; #1.04091.9025
Na ₃ VO ₄	1 mM	Sigma Aldrich; #S6508

Table 4: The table lists the individual components and their concentrations of the NP-40 lysis buffer used in the Receptor-Tyrosine Kinase Proteome Profiler™ Antibody Array (Cat#ARY001, R&D Systems). NP-40: (Octylphenoxy)- polyethoxyethanol, IGEPAL® CA-630; EDTA: Ethylenediaminetetraacetic acid; NaCl: sodium chloride; Na₃VO₄: sodium orthovanadate.

3.8 Western Blot

For the determination of protein amount in the cell lysate samples (see *Phospho-Profiling*) western blotting was performed. RPLP0, a ribosomal protein and actin, a cytoskeletal protein serve as appropriate candidates to compare lysate samples of cells cultured as a monolayer or as three-dimensional organoids.

3.8.1 Sample Preparation and SDS-PAGE

Lysate samples (see *Phospho-Profiling*) were removed from frozen storage (-20°C) and placed on ice. After heating up the thawed samples to 95°C for 5 min they were brought to RT to perform the SDS-PAGE. The electrophoresis was performed in vertical electrophoresis chambers (Mini-PROTEAN® Tetra Cell, Cat#165-8001). 25 µL of samples were loaded into each well of the polyacrylamide gel (10% TGX Mini-PROTEAN®, Bio-Rad, Cat#456-1033) and run at constant 60 mA for 40 – 45 min (TGS Running buffer, Bio-Rad, Cat#151-0772).

3.8.2 Western Blot

After SDS-PAGE the gel was sandwiched between ion reservoir stacks along with a 0.2 µm nitrocellulose membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad, Cat#170-4158). The assembly was placed between the anode of the cassette base of the Trans-Blot® Turbo Blotting System (Bio-Rad, Cat#170-415) and the cathode in the cassette lid. The proteins were transferred by applying 1.3A, 25V for 7 min using the Trans-Blot® System. After the transfer the blotting sandwich was disassembled and the gel was stained with Bio-Safe Coomassie Stain (Bio-Rad, Cat#161-0787) for 60 min on a rocking table.

The nitrocellulose membrane was washed with PBST, 1xPBS, (Life Technologies, Cat#10010-023) containing 0.05% Tween® 20 (Merck, Cat#8.22184.0500) and subsequently blocked with PBST containing 2% BSA for 30 min at RT on a rocking table. After a wash with PBST the primary antibody (Anti-RPLP0, 1:2000, Sigma Aldrich, Cat#AV40221; Anti-Actin, 1:200, Sigma Aldrich, Cat#A2066) was diluted in PBST containing 2% BSA, applied to the membrane and incubated for 1h at RT on a rocking table. After that the membrane was washed entirely with PBST and the secondary antibody (Anti-Rabbit IgG-HRP conjugate, 1:2000, DakoCytomation, Cat#P0448) was diluted in PBST containing 2% BSA, applied to the membrane and incubated for 1h at RT on a rocking table. After the secondary antibody incubation the membrane was washed with PBST and deionized water for subsequent data acquisition.

3.8.3 Chemiluminescence Detection

Chemiluminescent western blotting substrate (GE Healthcare, Cat#RPN2132) was prepared and added to the membranes. The signal was detected by the ChemiDoc XRS platform (Bio-Rad, Cat#170-8070) using the high sensitivity mode and analyzed with Quantity One Software.

3.9 Microscopy

Cell behavior and phenotypic changes of tumor and stromal cells were evaluated by confocal laser scanning microscopy and phase-contrast microscopy methods.

3.9.1 Confocal microscopy

Confocal laser scanning microscopy was performed using a Leica DMIRE2 microscope equipped with a TCS SP2 laser unit, EL6000 compact light source, CTR MIC electronics box and Leica Confocal Software Version 2.61 (Leica Microsystems, Heerbrugg, CH). The microscope was interfaced with argon, argon/krypton and helium/neon laser. Additionally, the microscope unit was equipped with an EL6000 compact light unit. This set-up covered an emission spectrum from 405 to 633 nm and was therefore suitable for the excitation of the fluorescent proteins mCFP (433 nm), EGFP (488 nm) and J-Red (584 nm) which were used for labeling the target cells. The fluorescent proteins' emission was detected by four photomultiplier tubes.

3.9.2 Phase-contrast microscopy

Phase-contrast microscopy was performed using a Leica DMI6000B inverted microscope equipped with CTR6500 microscope drive control, DFC420C digital microscope camera with a 5 Megapixel CCD sensor and Leica Application Suite Version 3.8.0 software.

3.10 Lentiviral Transduction

The lentiviral system LentiORF™ was purchased from Thermo Fisher Scientific, (formerly Open Biosystems) and consisted of five trans-lentiviral packaging plasmids, pTLA1-Pak, pTLA1-ENZ, pTLA1-ENV, pTLA1-Tat/Rev and pTLA1-TOFF (Figure 10) that supplied all viral proteins in trans and were required for the production of replication incompetent lentiviral particles. Furthermore the LentiORF™ system contained a transfection reagent (Express-In™), TLA-HEK293T packaging cell line, control vectors and ORF plasmid DNA. The fluorescent proteins, J-Red,

mCFP and EGFP were cloned into the multiple cloning site (MCS) of the pLEX empty vector. The EGFP expressing vectors were packaged into viral particles and stably transduced into the lung cancer cell lines HCC827, NCI-H1975 whereas primary human neonate dermal fibroblasts (nHDF) were lentivirally transduced to express J-Red or mCFP respectively.

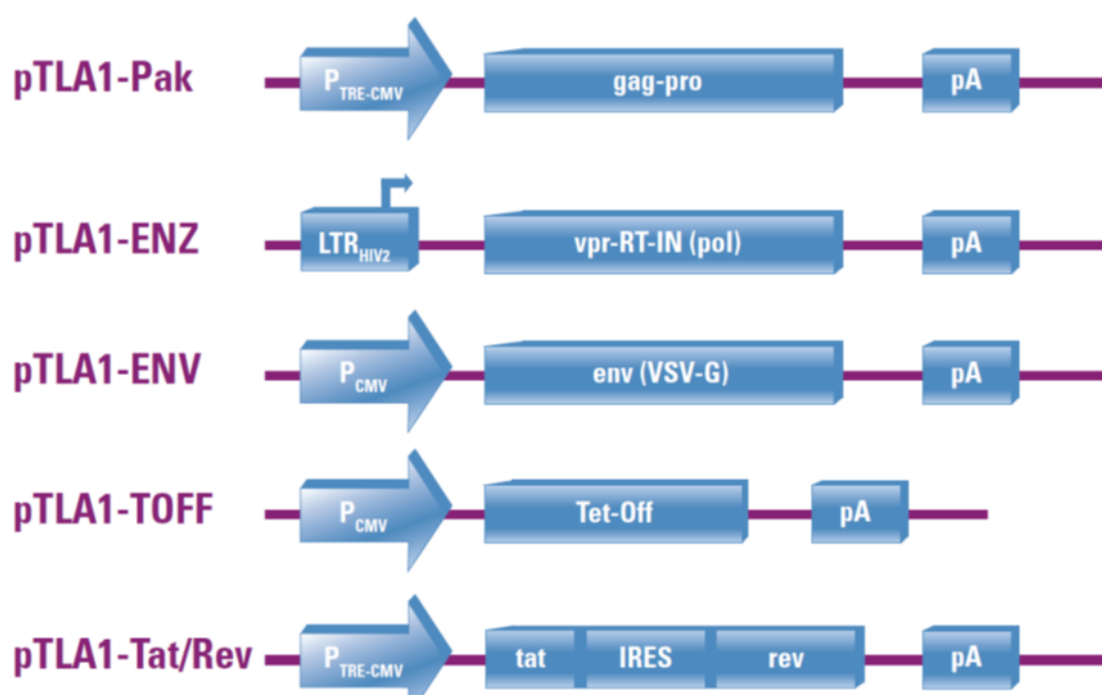


Figure 10: The packaging plasmids of pLEX System supply helper functions as well as structural and enzymatic proteins in trans to produce replication incompetent lentiviral particles.

pTLA1-Pak contains the *gag-pro* gene controlled under a strong TRE-CMV promoter. *Gag-pro* encodes structural proteins (*gag-pro*) essential for assembly and packaging the new virus within the packaging cell line. **pTLA1-ENZ** contains the accessory gene *vpr*, as well as the viral enzymes reverse transcriptase (RT) and integrase (IN). Genes encoding viral surface and transmembrane glycoproteins which are essential for the recognition and infection of the target cells are located on plasmid **pTLA1-ENV**. **pTLA1-TOFF** is required for the transcriptional activation of *Gag-pro* (**pTLA1-Pak**) and *Tat/Rev* (**pTLA1-Tat/Rev**) expression. **pTLA1-Tat/Rev** contains the viral genes *tat* and *rev* and includes an internal ribosomal entry site (*IRES*). *Tat* expression is required for transactivation of 5'LTRs whereas *rev* is necessary for the nuclear transport and the translation of mRNA transcripts and the full-length viral genome. (Image source: Thermo Scientific Open Biosystems)

The packaging cell line TLA-HEK293T was plated at a density of 5.5×10^6 cells per 100 mm culture dish in DMEM medium (Life technologies, Cat#11960-044). After o/n incubation at 37°C and 5% CO₂ in a humidified atmosphere the lipo-polymeric transfection reagent Express-In™ (Life Technologies, Cat#ETR4620) facilitated high

yield transfection of lentiviral vectors into packaging cells. The resulting lentiviral particles containing supernatant was diluted to 12.5% with appropriate growth medium to transduce target cells. Non-infected cells were eliminated using puromycin containing growth medium (1 µg/mL) for 2 weeks.

3.10.1 Fluorescent Proteins

Enhanced green fluorescent protein (EGFP), a modified *Aequorea*-derived fluorescent protein (AFP), is widely-used and emits green fluorescence at 509 nm when excited at 488 nm. The enhancement was accomplished by modifying GFP at two positions, i.e. F64L and S65T. It is a well characterized FP in terms of brightness, folding efficiency, photostability and cytotoxicity and represents therefore an optimal candidate. Monomeric cyan fluorescent protein (mCFP) emits blue fluorescent light at 475 nm when excited at 433 nm. Monomeric CFP comprises seven mutations relative to wtGFP, i.e. F64L, S65T, Y66W, N149I, M153T, V163A and A206K (Shaner NC et al., 2005). J-Red is a red fluorescent protein obtained by mutagenesis of a chromoprotein of an unidentified *Anthomedusae* jellyfish (Shagin DA et al., 2004) and emits red fluorescent light at 610 nm when excited at 584 nm.

4 Results

According to American Cancer Society the lifetime risk of developing cancer is 42%. Approximately the half of patients diagnosed with cancer will die of it. Hence, a primary area of contemporary medical research is the discovery and development of new anti-cancer drugs. Many advances have been produced by standard two-dimensional (2D) cell culture studies so far. Despite this progress recent data from 3D tumor model approaches indicate their decisive role in the evaluation of anti-cancer drugs (Bhadriraju K et al., 2002; Kim JB, 2005).

With approximately one third of all cancer deaths lung cancer accounts for more deaths than any other cancer type regardless of gender (American Cancer Society, 2011). Since non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers we focused on this major subclass. We developed different methods to generate cancer cell spheroids that are miniaturized 3D cell aggregates (tissue mimetics) that can form under specific experimental cell culture conditions. Each cancer cell line required a specific experimental set up to form coherent 3D spheroids. The typical work flow for the generation and subsequent analyses of homo- and heterotypic 3D cultures is depicted in Figure 11.

After generating spheroids with different experimental techniques we embedded them in natural or synthetic extracellular matrix (ECM) scaffolds and tested defined compositions of these ECM components with regard to their influence on tumor cell physiology. We phenotypically characterized human carcinoma cell lines in terms of their epithelial marker protein expression and differential proliferation when cultured in 2D or 3D. We already established an experimental workflow for the automatized generation of spheroids and organoids that might be amenable to high-throughput drug screening (Figure 11).

Work Flow for the Establishment of Tumor Mimetics

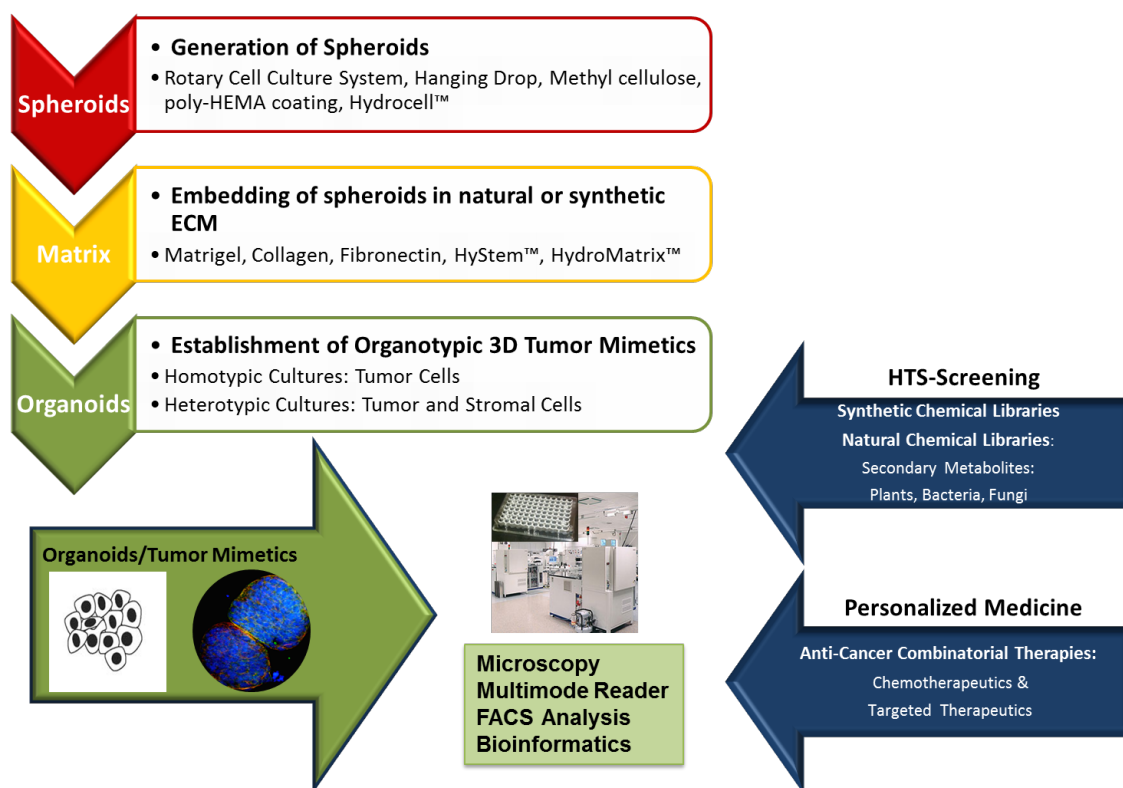


Figure 11: Generation of Tumor Mimetics.

The flow chart depicts a typical work flow for the generation of organoids, possible applications and analysis methods. Poly-HEMA, poly 2-Hydroxyethyl Methacrylate; ECM, Extracellular Matrix; HTS, High-throughput screening; FACS, Fluorescence activated cell sorting.

To characterize the cell physiology of organotypic 3D models in more detail we performed Affymetrix Gene Chips analyses. By utilizing microarray technology the transcriptome analyses revealed differences in transcriptome patterns of cells cultured in 2D or 3D. In addition Proteome Profiler™ Antibody Arrays were used to measure more than 40 different proteins simultaneously. These arrays allowed identifying differences on the protein level (i.e. phosphorylation patterns of cancer-related proteins) of cells grown in 2D or 3D. Moreover, it was demonstrated that drug response was significantly affected when cells were cultured as cell aggregates. The three-dimensionality of cell clusters and the physico-chemical cues of the surrounding microenvironment crucially influenced cell signaling and cell behavior and thereby drug sensitivity. It has been consistently shown that NSCLC frequently

overexpresses members of the erbB family, and that many NSCLC cells are “addicted” to signaling via mutated erbB family members (Riley GJ et al., 2006). Therefore we chose the listed cell lines on the basis of their mutations of the epidermal growth factor receptor HER1 to establish and validate a standardized 3D lung cancer model system (Table 5). For evaluating the ability of the 3D model to predict drug efficacy we tested EGFRIs (targeted anti-cancer agents) and conventional cytostatic therapeutics.

Beside matrix related effects it is known that cellular crosstalk between tumor and stromal cells is a crucial factor in cancer cell proliferation and invasion. By co-cultivating lung cancer cells with fibroblasts the effect of stromal cells on the cancer cells was shown. To discriminate microscopically between cancer cells and stromal cells both cell types were lentivirally transduced to stably express different fluorescent proteins.

4.1 Phenotypic Characterization of Lung Cancer Cell Lines

It has been consistently demonstrated that physiological cell signaling and related morphological and functional cellular changes rely crucially on the attachment of cells to surrounding three-dimensional mesh-like fibers rather than to two-dimensional coated surfaces (Cukierman E et al., 2002).

4.1.1 The used cell lines

The human lung cancer cell lines used in the experiments are fully genotyped. Selected cell line information including grading, HER1 and other mutations, type of malignancy and biological source of origin are listed in Table 5.

HCC827 cell line carries an E746-A750 deletion on exon 19 of the HER1 receptor. This hyperactivation mutation together with a 10-fold amplification of HER1 might lead to HER1 oncogene addiction in this cell line. This feature results in an increase in sensitivity towards HER1 inhibitors such as Gefitinib and Erlotinib.

In general, dramatic response to HER1 inhibitors may be ascribable to several HER1 mutations (Kosaka T et al., 2004). NCI-H1975 cell line carries a drug sensitive point mutation, L858R, on exon 21. It has been reported that this missense mutation increases the EGF induced activation of the HER1 two to three-fold compared to wild-type HER1 receptors; additionally this mutation caused an up to 12-fold prolonged activation of the mutant receptor. The localization of the L858R mutation near the receptor's ATP cleft causes a higher sensitivity towards HER1 inhibitors (Lynch TJ et al., 2004). In addition, this cell line carries a T790M point mutation on exon 20 which is associated with acquired drug resistance to Erlotinib or Gefitinib. This mutation impairs binding of the inhibitors to the tyrosine kinase domain. In contrast, CaLu-1 and NCI-H1437 cell lines express wild-type HER1 receptors.

Results/Phenotypic Characterization of Lung Cancer Cell Lines

Name	ATCC Cat. No.	Type	Tumor Stage	HER1 Mutations	Other Mutations	Source
HCC827	CRL-2868	Adenocarcinoma	n.a.	p.746_750del; 10x Ampl.	PIK3CA: p.N797fs; PIK3CB: p.H280fs; PIK3R1: p.P612fs; RB1: p.557_557del; TP53: p.P72R, p.V218del; Met: 2.2x	Primary, lung epithelial
NCI-H1975	CRL-5908	Adenocarcinoma	n.a.	p.T790M, p. L858R	TP53: p.P72R, p.R273H; PIK3CA: p.G118D; Met: 2.7x	Primary, lung epithelial
CaLu-1	HTB-54	Epidermoid Carcinoma	Grade III	wt	KRAS: p.G12C	Metastatic, pleura
NCI-H1437	CRL-5872	Adenocarcinoma	Grade I	wt	CDKN2a (p14ARF): c.471_471del; c.522_522del TP53: p.R267P	Metastatic, pleural effusion

Table 5: Background information of used human lung cancer cell lines.

The table provides information on two HER1 mutant and two HER1 wild-type NSCLC cell lines, including repository number, type of malignancy, grading, EGFR (HER1) and other selected mutations and the biological source.

We phenotypically characterized these human lung cancer cell lines via phase-contrast and confocal laser scanning microscopy (Figure 12). To determine epithelial cell polarity and associated differentiation status of the carcinoma cell lines the cells were fixed in 2.5% formaldehyde for 30 minutes and processed for immunofluorescence microscopy.

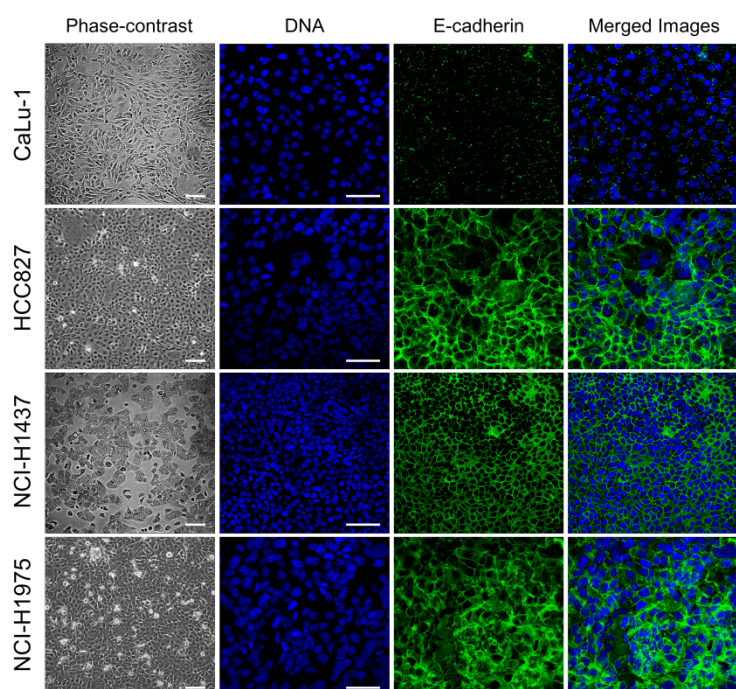


Figure 12: Morphology and expression of cell-polarity marker E-cadherin in human lung cancer cell lines grown as monolayers.

Phase-contrast (left panel) and confocal laser scanning micrographs (right panel) show morphology and the distribution of E-cadherin in formaldehyde fixed cells after immunofluorescent staining. All investigated cell lines show high E-cadherin expression except CaLu-1 which feature a highly unpolarized phenotype (mesenchymal phenotype). HCC827, NCI-H1437 and NCI-H1975 exhibit typical epithelial characteristics in terms of morphology and E-cadherin expression. Bars, 75 μ m.

We could demonstrate that all used lung cancer cell lines except CaLu-1 highly expressed E-cadherin. HCC827, NCI-H1437 and NCI-H1975 cells showed typical epithelial characteristics. Therefore CaLu-1 cells adopted a mesenchymal, dedifferentiated morphology that correlated with phase-contrast micrographs (Figure 12). It has been demonstrated that during EMT the loss of E-cadherin expression is concomitant with *de novo* vimentin expression (Eger et al., 2005). We could show that CaLu-1 cells highly up-regulated the type III intermediate filament protein vimentin (Figure 13).

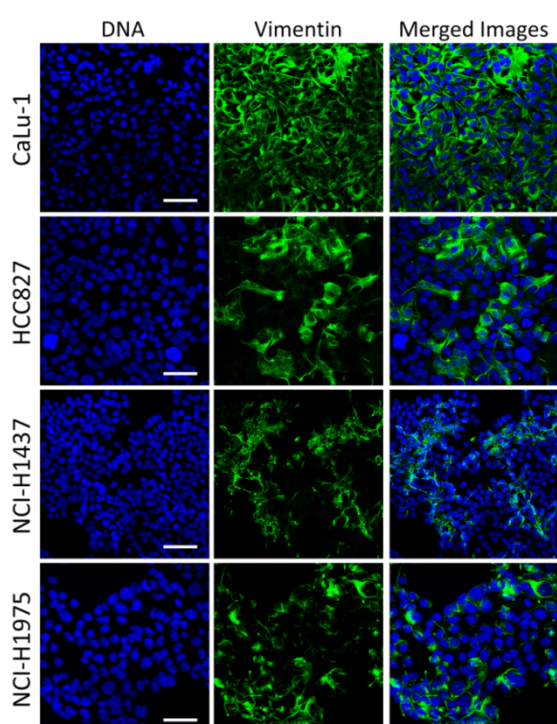


Figure 13: Expression of intermediate filament protein vimentin in human lung cancer cell lines grown as monolayers.

Confocal laser scanning micrographs of horizontal optical sections show the distribution of vimentin in formaldehyde fixed cells after immunofluorescent staining. All investigated cell lines show basal vimentin expression except CaLu-1 which feature a highly up-regulated vimentin expression. This leads beside other molecular mechanisms to the acquisition of a mesenchymal phenotype. HCC827, NCI-H1437 and NCI-H1975 feature typical epithelial characteristics. Bars, 75 μ m.

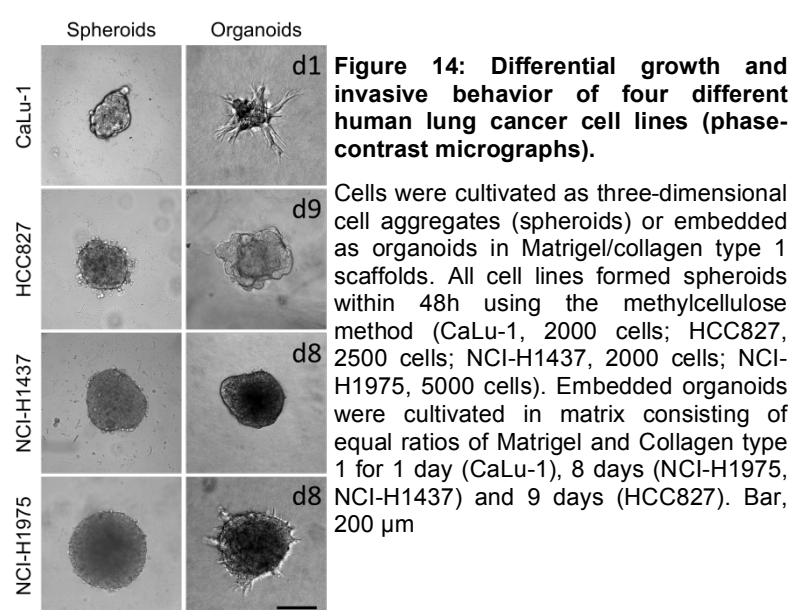
The different lung cancer cell lines needed individually defined conditions to form spheroids using various methods. While NCI-H1975 and NCI-H1437 spheroids feature generally high stability and a smooth rim single invading single HCC827 and CaLu-1 cells are visible at the spheroid edge. Even though all used cell lines originate from epithelium, in a monolayer culture NCI-H1975 and especially CaLu-1

cells feature a rather mesenchymal phenotype. HCC827 and NCI-H1437 cells exhibit typical epithelial polarization and a cobblestone appearance.

The immunofluorescence microscopy revealed the differential expression of EMT associated markers in the carcinoma cell lines. In CaLu-1 cells the downregulation of E-cadherin expression is concomitant with the de novo expression of vimentin. The loss of adhesive function of E-cadherin may promote the dedifferentiation of epithelial cells to an invasive stage. All data considered, CaLu-1 cells are highly undifferentiated carcinoma cells expressing weak cell-cell junction proteins and are therefore more motile (Frixen UH et al., 1991). This may lead to a rather mesenchymal and invasive phenotype.

4.1.2 Human Lung Cancer Cell Lines embedded in ECM as Organoids: Phenotypic Characterization using Phase-contrast Microscopy

As illustrated in Figure 14 upon cultivation as organoids the surrounding ECM affects the microtumors' proliferation, growth and in particular the invasive behavior. CaLu-1 cells invaded already on day one the Matrigel-collagen type 1 matrix, followed by NCI-H1975 on day 8. Despite a small increase in diameter of the HCC827 microtumors invasion was not observed for the cell lines HCC827 and NCI-H1437.



A direct comparison of different ECM protein compositions revealed the physico-chemical and biological impact of the matrix on the embedded spheroids, here referred as organoids. As an example two highly differentiated and mesenchymal lung cancer cell lines are shown. Even though all depicted cell lines originate from human lung epithelial malignancies the organoids showed significant differences in growth and invasion behavior. Spheroids of all four lung cancer cell lines were generated using poly-HEMA coated 96-well plates. Spheroids were harvested on the second day and embedded into different natural ECM scaffolds. These organoids were incubated then at 37°C and 5% CO₂ in a humidified atmosphere for up to 11 days. Images were acquired by phase-contrast microscopy.

CaLu-1

Matrigel™

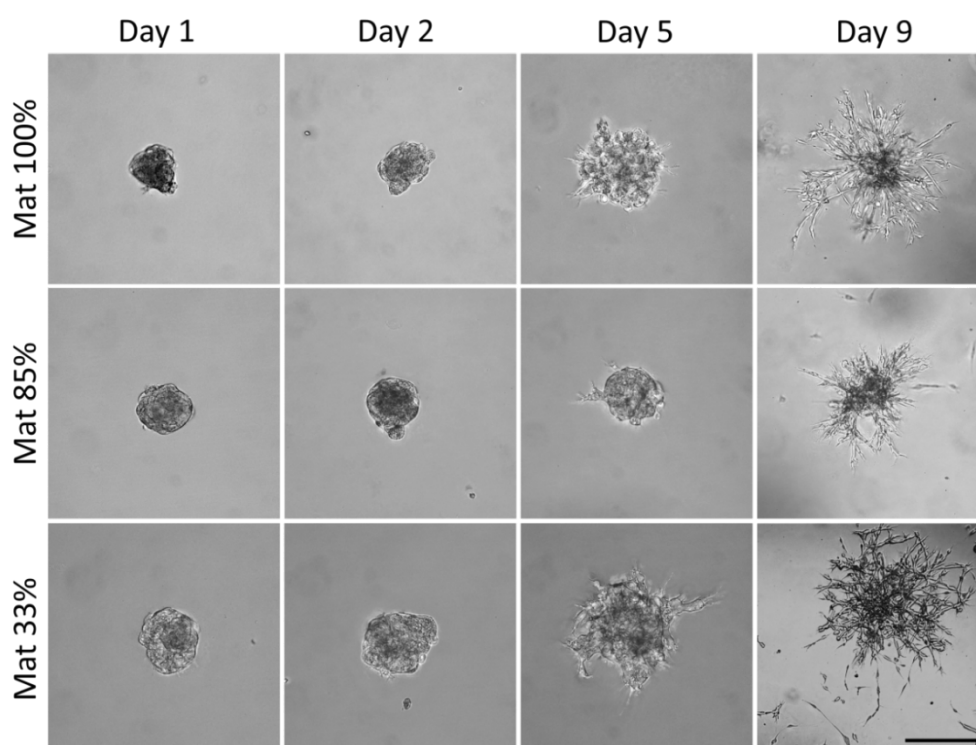


Figure 15: Growth and invasive behavior of human lung cancer cell line CaLu-1 in Matrigel matrices (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in different matrices consisting of 33% and 85% v/v Matrigel diluted with growth medium or in undiluted (100% v/v) Matrigel matrix. Organoids were cultivated for 9 days under common culture conditions. An increase in Matrigel concentration correlated with higher invasiveness of the microtumors on day 9. Bar, 200 µm.

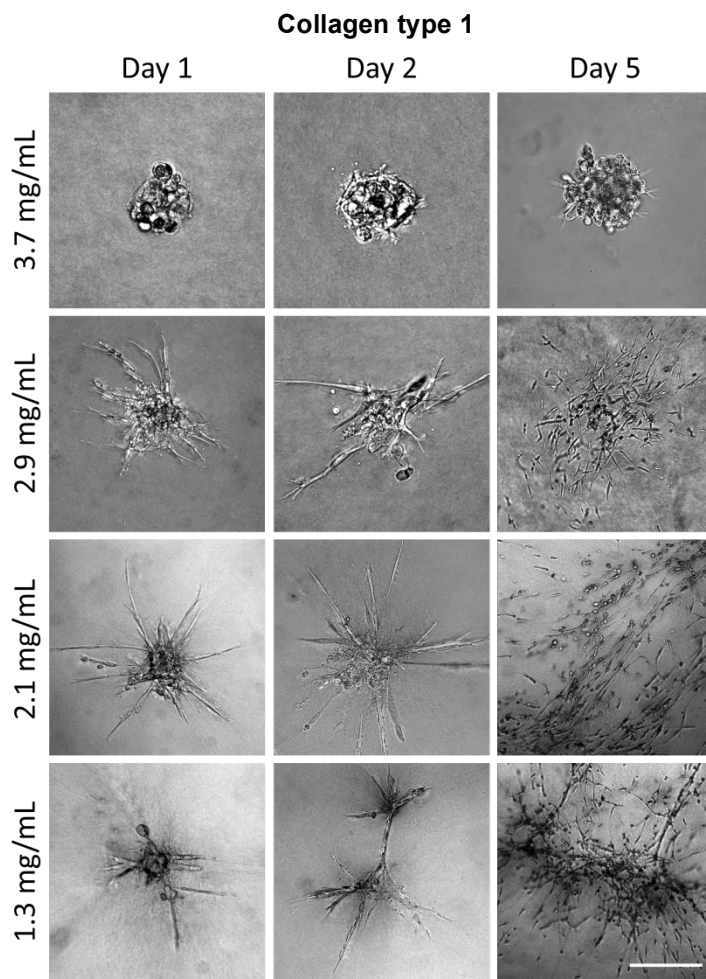


Figure 16: Growth and invasive behavior of human lung cancer cell line CaLu-1 in collagen Type 1 matrices (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in different matrices consisting of 1.3 – 2.9 mg/mL collagen type 1 concentration diluted with growth medium; or in undiluted collagen matrix (3.7 mg/mL). Organoids were cultivated for 5 days under common culture conditions. Invasion of microtumors could be observed at collagen concentrations between 1.3 - 2.9 mg/mL. Bar, 200 μ m.

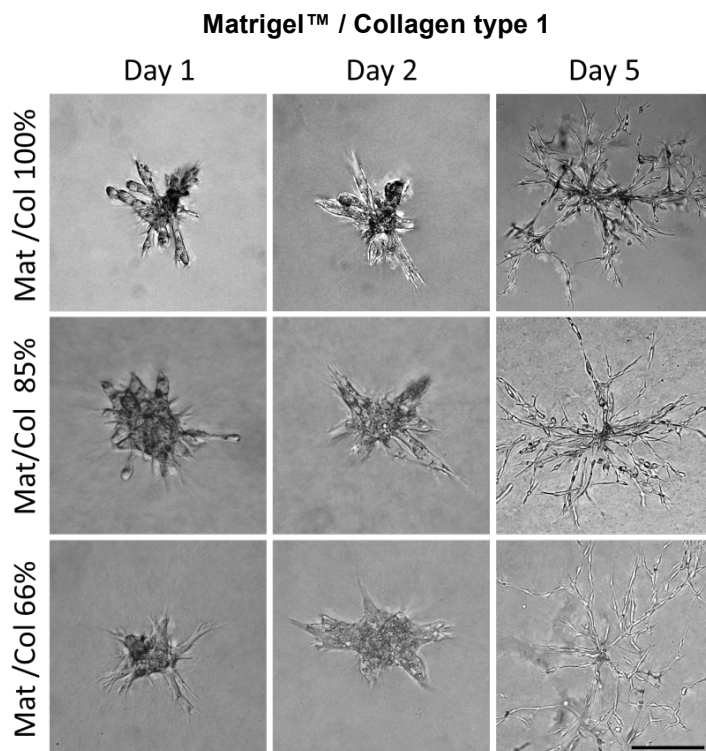


Figure 17: Growth and invasive behavior of human lung cancer cell line CaLu-1 in Matrigel/collagen Type 1 matrices (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded into Matrigel/collagen type 1 matrices consisting of 66% and 85% v/v Matrigel/collagen matrix diluted with growth medium; or undiluted (100% v/v) Matrigel/collagen matrix and cultivated for 5 days under common culture conditions. The matrix composed of equal amounts of Matrigel and collagen. Invasion of microtumors could be observed at all matrix concentrations. Bar, 200 μ m.

CaLu-1 organoids were able to invade into all ECM scaffolds regardless of composition except 3.7 mg/mL collagen type 1 concentration. When CaLu-1 organoids are embedded into Matrigel matrix they showed different invasion behavior depending on the matrix composition. In collagen type 1 matrices CaLu-1 organoids exhibited the highest invasion rate in all concentrations between 1.3 and 2.9 mg/mL collagen already on day 1. In matrices composing of Matrigel/collagen type 1 mixtures CaLu-1 organoids showed invasion characteristics similar to collagen type 1 matrices (Figures 15-17).

HCC827

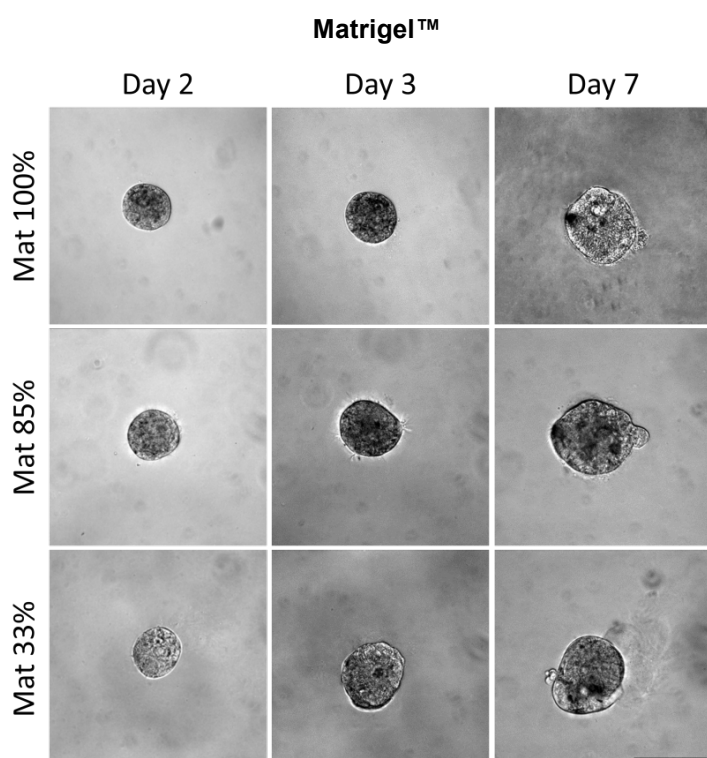


Figure 18: Growth and invasive behavior of human lung cancer cell line HCC827 in Matrigel matrices (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in different matrices consisting of 33% and 85% v/v Matrigel diluted with growth medium or in undiluted (100% v/v) Matrigel matrix. Organoids were cultivated for 7 days under common culture conditions. Bar, 200 μ m.

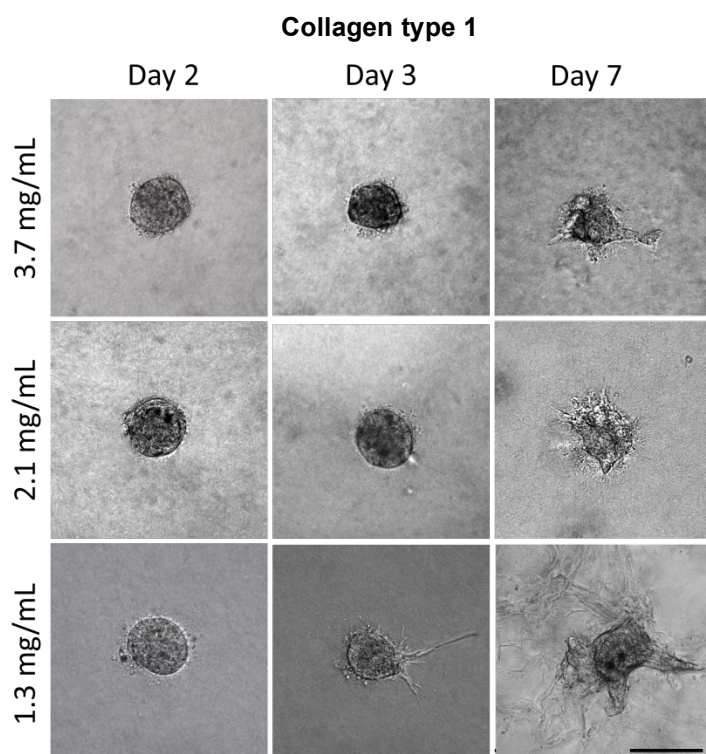


Figure 19: Growth and invasive behavior of human lung cancer cell line HCC827 in collagen type 1 matrices (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in different matrices consisting of 1.3 – 2.9 mg/mL collagen type 1 concentration diluted with growth medium; or in undiluted collagen matrix (3.7 mg/mL). Organoids were cultivated for 7 days under common culture conditions. Bar, 200 μ m.

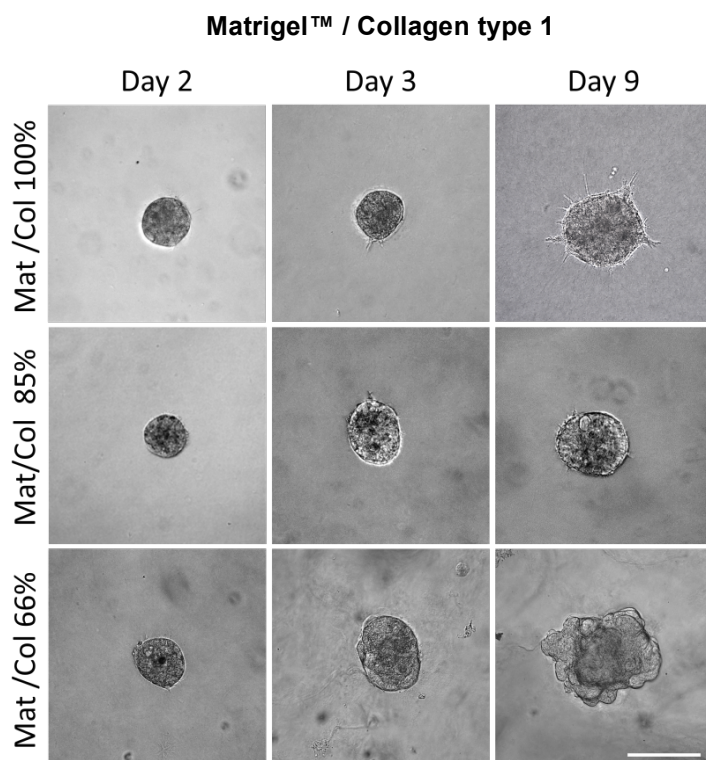


Figure 20: Growth and invasive behavior of human lung cancer cell line CaLu-1 in Matrigel/collagen Type 1 matrices (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded into Matrigel/collagen type 1 matrices consisting of 66% and 85% v/v Matrigel/collagen matrix diluted with growth medium; or undiluted (100% v/v) Matrigel/collagen matrix and cultivated for 5 days under common culture conditions. The matrix composed of equal amounts of Matrigel and collagen. Invasion of microtumors could be observed at all matrix concentrations but the morphology of the invasive cells was different. Bar, 200 μ m.

HCC827 organoids were only able to weakly invade into ECM scaffolds consisting of collagen type 1 protein. Embedded into Matrigel and Matrigel/collagen type 1 matrices, HCC827 organoids showed no significant invasive behavior. In collagen type 1 matrices HCC827 organoids showed the highest invasion rate in all concentrations between 1.3 and 2.9 mg/mL collagen (Figures 18-20). HCC827 organoids were able to grow in all matrices regardless of composition.

NCI-H1437

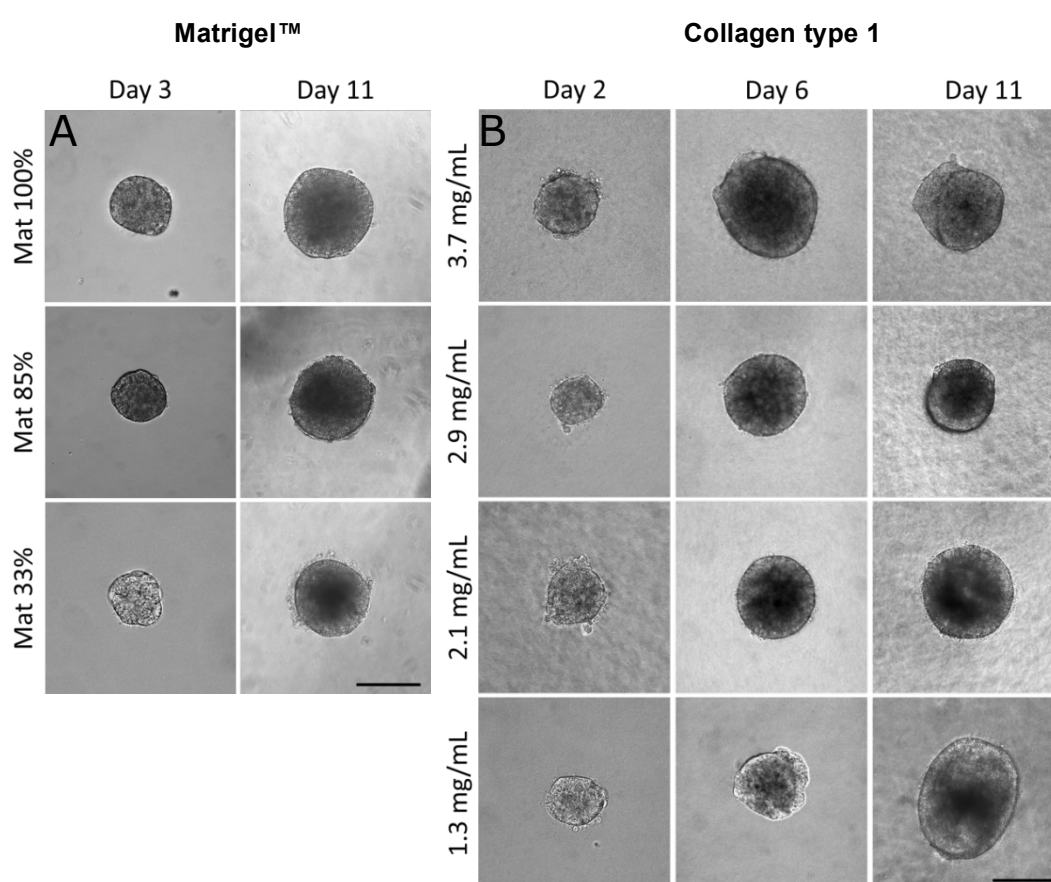
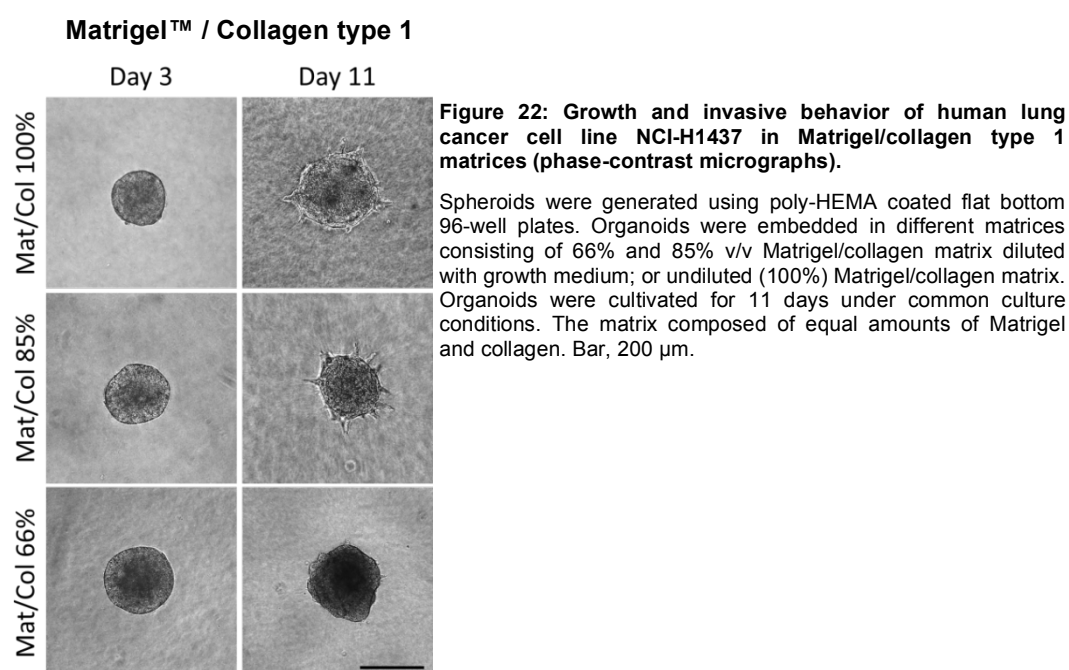


Figure 21: Growth and invasive behavior of human lung cancer cell line NCI-H1437 in Matrigel matrices [Panel A], and collagen type 1 matrices [Panel B] (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in different matrices consisting of 33% and 85% v/v Matrigel diluted with growth medium or in undiluted Matrigel matrix (A) and in collagen type 1 matrices consisting of 1.3 – 2.9 mg/mL collagen type 1 concentration (B) diluted with growth medium; or in undiluted collagen matrix (3.7 mg/mL). Organoids were cultivated for 11 days under common culture conditions. Bar, 200 μ m.



NCI-H1437 organoids were only able to invade into ECM scaffolds consisting of 85% v/v Matrigel/collagen matrix diluted with growth medium; and undiluted (100% v/v) Matrigel/collagen matrix. Embedded into Matrigel or collagen type 1 matrices, NCI-H1437 organoids showed no invasive behavior (Figures 21, 22). NCI-H1437 organoids were able to grow in all matrices regardless of composition.

NCI-H1975

NCI-H1975 organoids were able to invade into ECM scaffolds consisting of collagen type 1 protein. Embedded into matrices consisting of 85% v/v Matrigel/collagen matrix diluted with growth medium; and undiluted (100% v/v) Matrigel/collagen matrices NCI-H1975 organoids showed moderate invasive behavior (Figures 23, 24). In matrices consisting of 66% v/v Matrigel/collagen, NCI-H1975 organoids exhibited the most significant increase in average diameter.

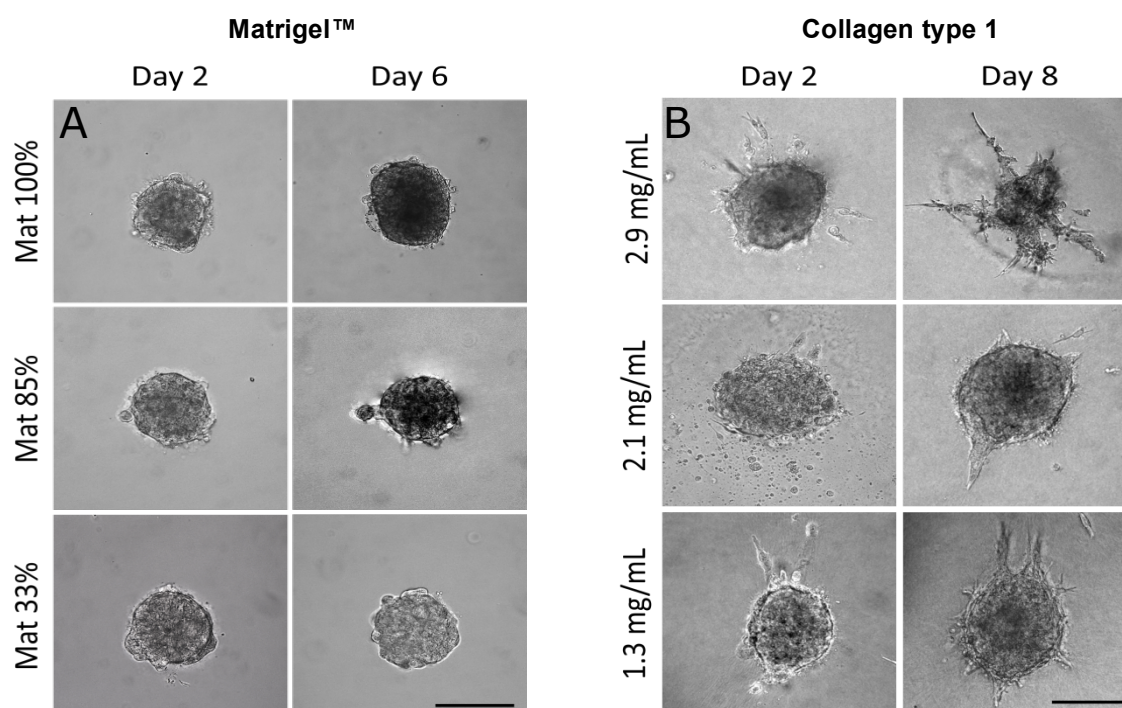


Figure 23: Growth and invasive behavior of human lung cancer cell line NCI-H1975 in Matrigel matrices [Panel A], and collagen type 1 matrices [Panel B] (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in different matrices consisting of 33% and 85% v/v Matrigel diluted with growth medium or in undiluted Matrigel matrix (A) and in collagen type 1 matrices consisting of 1.3 – 2.9 mg/mL collagen type 1 concentration (B) diluted with growth medium; or in undiluted collagen matrix (3.7 mg/mL). Organoids were cultivated for 6 (A) or 8 (B) days under common culture conditions. Bar, 200 µm.

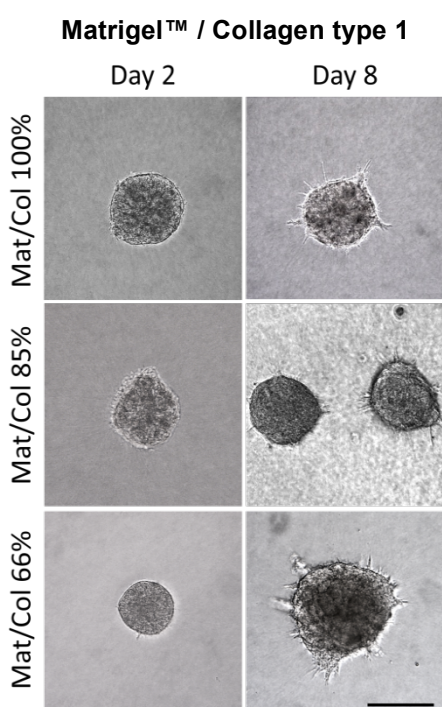


Figure 24: Growth and invasive behavior of human lung cancer cell line NCI-H1975 in Matrigel/collagen type 1 matrices (phase-contrast micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in different matrices consisting of 66% and 85% v/v Matrigel/collagen matrix diluted with growth medium; or undiluted (100% v/v) Matrigel/collagen matrix. Organoids were cultivated for 8 days under common culture conditions. The matrix composed of equal amounts of Matrigel and collagen. Bar, 200 µm.

4.1.3 Marker Protein Expression in 3D: Phenotypic Characterization using Confocal Laser Scanning Microscopy

So far we have characterized the lung cancer cell lines in terms of morphology and expression of epithelial differentiation markers when grown two-dimensionally. Additionally, we investigated the growth and invasion behavior of multi cellular tumor organoids (MCTO) embedded into different natural ECM scaffolds.

To determine vitality and quality of cells within MCTOs we examined the expression of several epithelial markers. In addition we localized the regions of cell proliferation within organoids using Ki67 as a proliferation marker. For this we generated spheroids using the methylcellulose method. The matrix consisting of a mixture of Matrigel and collagen was evaluated in previous experiments as an ideal matrix for the majority of cell lines. Therefore the organoids were embedded in this matrix, fixed in 3% formaldehyde and processed for immunofluorescence microscopy.

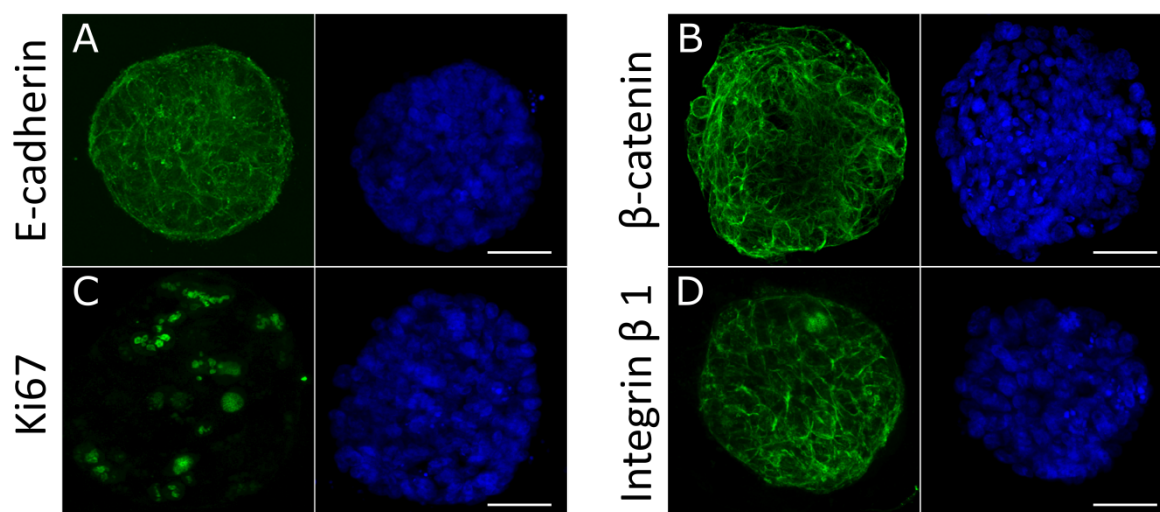


Figure 25: Marker protein expression of human lung cancer cell line HCC827 in Matrigel/collagen type 1 matrices (confocal laser scanning micrographs).

Spheroids were generated using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded in Matrigel/Collagen matrix consisting of 66% v/v Matrigel/collagen matrix diluted with growth medium. Confocal laser scanning micrographs of horizontal optical sections show the distribution of E-cadherin, β -catenin, Ki67 and integrin β 1 in formaldehyde fixed organoids after immunofluorescent staining. Bars, 75 μ m.

We could show in immunofluorescent (IF) micrographs that the cells within organoids are alive and show typical marker expression. This finding correlates with previous apoptosis assays using Annexin V (Kalod R, 2011). The immunostaining allowed also for marker staining of cells residing in the very center of organoids larger than 200 μm in diameter (Figure 25). We therefore could demonstrate that IF staining might be a suitable method to detect alterations in protein expression, apoptosis and necrosis that may result from drug treatment.

4.2 RNA Expression Profiling in 2D vs. 3D: Affymetrix Gene Chip Technology

The pattern of gene expression within a cell determines the cell's phenotype and function. Exploiting cDNA microarray technology provided insight of global gene expression of the NSCLC cell lines CaLu-1, HCC827, NCI-H1437 and NCI-H1975. We compared the cell lines' transcriptomes when grown as a monolayer with those of organoids embedded in Matrigel/collagen type I matrices. The composition of the used matrix was evaluated in previous experiments for all cell lines. This was determined with regard to growth, proliferation and invasion of the embedded organoids.

For 2D RNA samples cells were seeded before RNA preparation onto 100 mm culture dishes. For 3D RNA samples the spheroids were generated using the rotary cell culture bioreactor. For RNA preparation the QIAgen's miRNeasy Mini Kit containing Trizol was used. RNA quality determination via capillary gel electrophoresis using the Experion™ Platform (Bio-Rad, Figure 26) was performed and the RNA samples were frozen (-80°C) until further processing. After establishing cDNA from the RNA samples the expression profiling was performed using Affymetrix gene chips (Affymetrix Inc., Table 6 A, B).

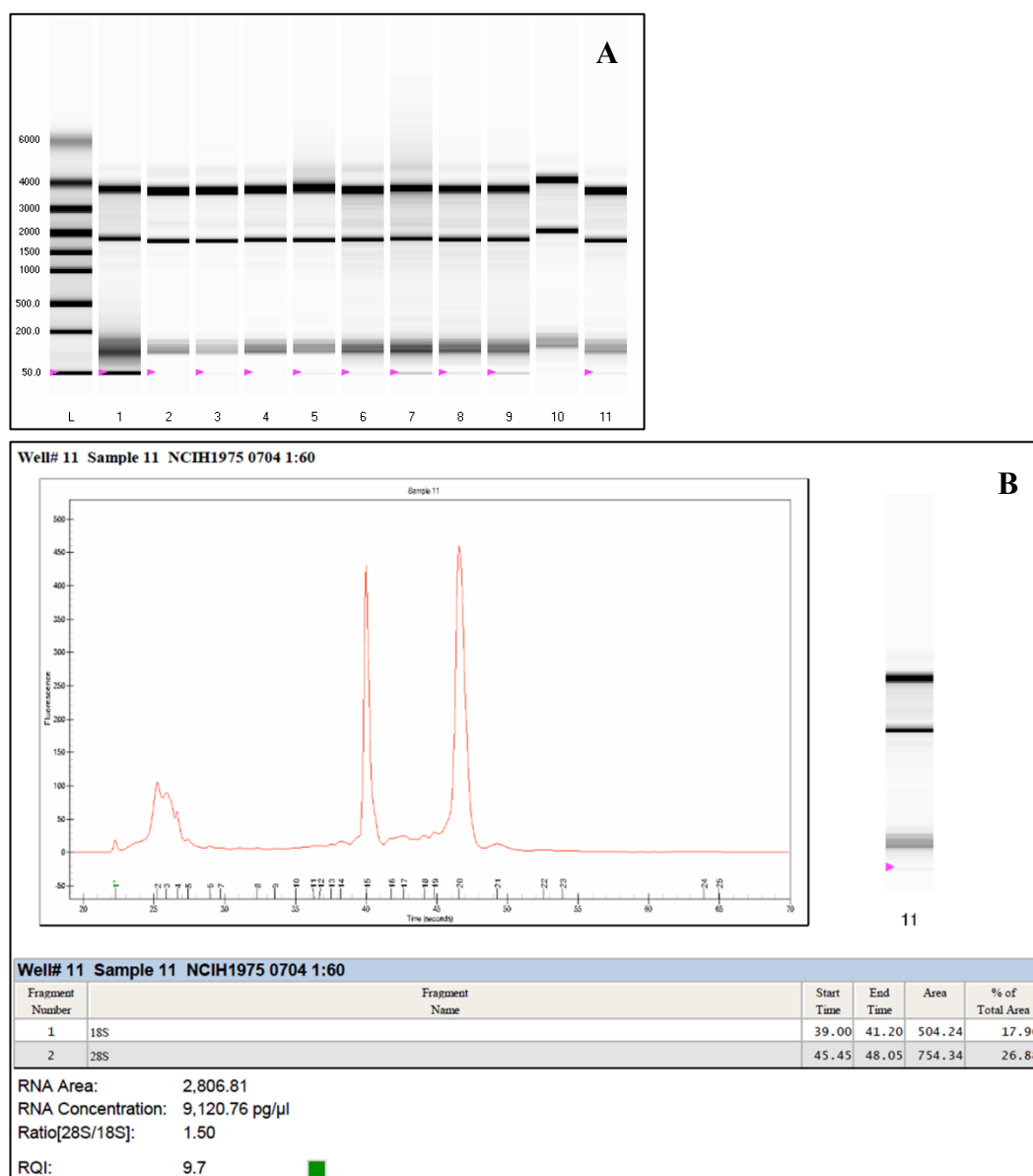


Figure 26: Preliminary RNA quality tests using the capillary gel electrophoresis platform Experion™ and Experion RNA StdSens Analysis Kit (BioRad).

A| Virtual gel showing RNA bands of different dilutions of 2D and 3D cell lysates of HCC827 and NCI-H1975 cell lines.

B| Electropherogram of a 3D sample (NCI-H1975, Matrigel/Collagen) with a lower marker peak at 22sec, a 5S rRNA peak at 25sec, a 18S rRNA peak at 40sec and a 28S rRNA peak at 47sec. The results table below includes the RNA concentration and the ratio between 28S and 18S as an indicator for RNA integrity (theoretically ≤ 2.7 , normally 1.2-1.95). The **RNA Quality Indicator (RQI)** allows to standardize and quantitate RNA integrity (10 "intact" to 1 "fully degraded").

A HCC827 2D on Matrigel Coating vs. 3D in Matrigel/Collagen Matrix

Gene Name	Localisation	Fold Change	
serum amyloid A1	11p15.1	28,1	3D higher than 2D
small nucleolar RNA, C/D box 61	Xq26.3	9,1	
lipocalin 2 (oncogene 24p3)	9q34	9,0	
ceruloplasmin (ferroxidase)	3q23-q25	8,1	
V-set and immunoglobulin domain containing 1	Xq22.3	7,9	
chemokine (C-C motif) ligand 20	2q33-q37	7,3	
complement factor H	1q32	7,0	
complement factor B	6p21.3	6,9	
interferon, alpha-inducible protein 27	14q32	6,1	
transcobalamin I (vitamin B12 binding protein, R binder family)	11q11-q12	6,0	
small nucleolar RNA, C/D box 38B	1p34.1-p32	8,3	2D higher than 3D
small nucleolar RNA, C/D box 59B	12q13.3	5,7	
kallikrein-related peptidase 6	19q13.3	5,0	
bicaudal C homolog 1 (Drosophila)	10q21.1	5,1	
small nucleolar RNA, C/D box 117	6p21.33	4,6	
pleckstrin homology-like domain, family B, member 2	3q13.2	4,5	
ribosomal protein SA	3p22.2	4,5	
erythrocyte membrane protein band 4.1-like 2	6q23	4,4	
small nucleolar RNA, C/D box 28	11q13	4,4	
small nucleolar RNA, C/D box 25	11q13	4,3	

B NCI-H1975 2D on Matrigel Coating 2D vs. 3D in Matrigel/Collagen Matrix

Gene Name	Localisation	Fold Change	
small nucleolar RNA, H/ACA box 75	2q37.1	5,0	3D higher than 2D
ribonuclease, RNase A family, 4	14q11.1	4,5	
similar to cleavage stimulation factor, 3 pre-RNA, subunit 2	-	4,1	
serpin peptidase inhibitor, clade B (ovalbumin), member 4	18q21.3	3,8	
matrix metalloproteinase 13 (collagenase 3)	11q22.3	3,8	
triggering receptor expressed on myeloid cells 1	6p21.1	3,6	
stanniocalcin 1	8p21-p11.2	3,6	
ADAM metalloproteinase domain 28	8p21.2	3,2	
serpin peptidase inhibitor, clade A (antitrypsin), member 3	14q32.1	3,2	
angiogenin, ribonuclease, RNase A family, 5	14q11.1-q11.2	3,2	
putative binding protein 7a5	7p15.3	5,2	2D higher than 3D
small nucleolar RNA, C/D box 28	11q13	3,9	
similar to 60S ribosomal protein L22 (Heparin-binding protein HBp15)	2q13	3,8	
alanyl (membrane) aminopeptidase (aminopeptidase N)	15q25-q26	3,7	
small nucleolar RNA, C/D box 18A	15q22	3,5	
small nucleolar RNA, C/D box 26	11q13	3,4	
RNA, small nucleolar	11q23.3-q25	3,4	
small nucleolar RNA, C/D box 56	20p13	3,2	
small nucleolar RNA, H/ACA box 29	6q25.3	3,2	
similar to nuclear protein in testis	10q23.2	3,2	

Table 6: Selected changes in gene expression of HCC827 and NCI-H1975 lung cancer cell lines cultured as a monolayer on Matrigel coating or as MCTOs in Matrigel/Collagen Matrix.

The list contains ten differentially expressed genes, and their chromosomal localisation, in HCC827 cells (A) and NCI-H1975 cells (B) arranged according to their fold change.

The GeneChip human mapping assay (Affymetrix) was used to provide genome-wide data on gene expression patterns. We investigated how gene expression in the lung adenocarcinoma cell lines HCC827 and NCI-H1975 is altered due to 3D

cultivation. The expression of approximately 35.500 genes was analyzed and ten differentially expressed genes are boxed (Table 6 A, B).

Microarray Analysis

The highest differentially expressed gene encodes for serum amyloid A 1 (SAA1), an apolipoprotein which is produced in the liver. We could show a 28-fold up-regulation of SAA1 in MCTOs of adenocarcinoma cell line HCC827 compared to monolayer culture on Matrigel coated plates. An up-regulation of this inflammatory protein in renal cell carcinomas was reported to increase the expression of metalloproteinase (MMP)-9 and hence the invasive behavior of tumor cells (Paret C et al., 2010).

Lipocalin 2 (NGAL) plays an important role in the transport of lipophilic ligands such as progesterone and prostaglandin. It is part of MMP-9 complex in human neutrophils where it protects MMP-9 and preserves MMP-9 activity. NGAL mediates inflammation, is up-regulated in many cancers and was reported to stimulate tumor growth in breast cancer (Fernández CA et al., 2005). We found a 9-fold up-regulation of Lipocalin 2 in HCC827 MCTOs compared to monolayer cultured cells.

Genes encoding for immunomodulatory proteins that serve as protectors from complement mediated lysis, such as complement factor B and H, are up-regulated (7-fold) in MCTOs of HCC827 cell line.

Ovalbumin, a peptidase inhibitor, is 4-fold up-regulated in NCI-H1975 MCTOs compared to monolayer culture. This inhibitor is often highly expressed in tumors originating from GI tract, the lung and thyroid. 30-50% of adenocarcinomas strongly express ovalbumin (Heighway J et al., 2004).

Beside altered expression of protein encoding genes a remarkable number of genes encoding for small nucleolar RNAs (snoRNAs) are differentially expressed in MCTOs compared to monolayer cultured cells. Since snoRNAs are non-coding RNAs that might regulate cell fate they could consequently contribute to oncogenesis as reviewed very recently (Williams GT and Farzaneh F, 2012). Apart from their known function as guide RNAs for the post-transcriptional modification of ribosomal RNAs, only little is known about their role in diseases like cancer. The EGFR expression in HCC827 cells which carry a 10-fold amplification mutation is 2-fold higher in

monolayer cell culture than in organoids. No significant difference in EGFR gene expression was observed for the NCI-H1975 cell line.

Upcoming pathway analyses using the Ingenuity software (Ingenuity Systems) will help to decipher signaling pathways specifically activated or inhibited in 3D.

4.3 Differential Phosphorylation Patterns in 2D vs. 3D: Proteome Profiler Antibody Array™

A frequent reversible post-translational modification that plays a central role in tumorigenesis is phosphorylation. For this purpose it is essential to determine the phosphorylation patterns of crucial signaling molecules associated in tumorigenesis (Mann M and Jensen ON, 2003).

By using Proteome Profiler Antibody Arrays™ (R&D Systems) we determined the phosphorylation state of proteins involved in:

- RTK signaling
- MAPK signaling
- Phosphokinase signaling
- Apoptosis

We compared the cell lines' phosphorylation patterns when grown as a monolayer with those of organoids embedded in Matrigel/collagen type I matrices.

For 2D samples cells were seeded before phospho-profiling onto 100 mm culture dishes. For 3D samples, spheroids were generated using poly-HEMA coated 96-well plates and embedded in Matrigel/collagen Type 1 matrices. The cells and organoids were processed for phospho-proteome analyses according to manufacturer's instructions (see Material and Methods). The cells were lysed, added to the membranes and incubated. The secondary anti-phospho-tyrosine-HRP antibody was added and chemiluminescent western blotting substrate was applied. The signal was detected by a ChemiDoc XRS platform. For normalization of cell number western blots against actin and RPLP0 were performed with 2D and 3D lysates. For CaLu-1 cells it was additionally normalized for total EGFR which brought the same result as actin.



A| Phosphorylated proteins in the lysates of cells grown as a monolayer (2D) or organoids embedded in Matrigel/collagen matrices (3D) were detected on antibody arrays via chemiluminescence. **B|** Relative levels of tyrosine phosphorylation of RTK related proteins in 2D and 3D. RTKs are significantly more abundant in 3D culture compared to cell monolayer. **C|** Actin for normalization of cell numbers in 2D and 3D.

Data from RTK array are shown in Figure 27. For normalization of cell number western blots against actin and RPLP0 were assessed in 2D and 3D lysates. For CaLu-1 also total EGFR levels were measured for normalization which showed same results. We could demonstrate that the phosphorylation of RTK related proteins is higher in organoids compared to cell monolayer. Exclusively in lysates of the cell line NCI-H1975 a relatively balanced 2D/3D expression of phospho-proteins was observed. The crucial role of epidermal growth factor receptor 1 (EGFR, ErbB1) in lung cancer has been discussed earlier. RTK Antibody Arrays™ revealed a significant overexpression (47-fold) of a phosphorylated form of this receptor in CaLu-1 organoids compared to conventional cell monolayer. This finding correlates strongly with the proliferative and metastatic potential of this cell line as MCTOs that was already determined microscopically. Furthermore we detected higher levels of pEGFRs in organoids in all other investigated cell lines as well (2-fold NCI-H1975; 9-fold HCC827; 13-fold NCI-H1437). While ErbB1 binds ligands (e.g. EGF, TGF- α , Amphiregulin) and directly relays signals via autophosphorylation of its intracellular domain into the cell ErbB2 and ErbB3 are non-autonomous. ErbB2 lacks the capacity to bind a growth-factor ligand and ErbB3 feature a defective kinase activity. Despite this fact, ErbB2 and ErbB3 form heterodimeric complexes with other family members to generate ErbB signaling (Citri A and Yarden Y, 2006). We could demonstrate that ErbB2 and ErbB3 were highly phosphorylated in MCTOs. It was reported that hyperactivated EGFR signaling mediates anti-apoptotic and pro-survival signals through Ras-Raf-MEK, ERK1 and ERK2, PI3K–Akt and STAT3 and STAT5 pathways (Sharma SV et al., 2007). We could demonstrate that MEK1/2, ERK1, Akt and STAT3/5 are significantly more phosphorylated in MCTOs of different cell lines than in monolayer cultures (Table 7). It has been reported that STAT3, regulate genes for anti-apoptosis, pro-proliferation, angiogenesis, and metastasis (Yu H et al., 2009). We determined an 11-fold increase of STAT3 Y705 in NCI-H1437 MCTOs compared to conventional monolayer.

The phosphoinositide signaling system is a crucial regulator of most cellular processes. In this system phospholipase C gamma (PLC γ) acts as a key enzyme in the phosphoinositide signaling, especially in cancer. In mammary tumor cells it has

been demonstrated that PLC γ regulates cancer cell motility under the control of EGF (Bunney TD and Katan M, 2010). We detected an 18-fold increase of PLC γ 1 phosphorylation at Y783 in the NCI-H1437 cell line when cultured as MCTOs.

A comprehensive overview of phospho-proteome profiles of the four lung cancer cell lines in 2D and 3D is depicted below (Table 7).

Receptor Tyrosine Kinase Phosphoarray A				
	CaLu-1	HCC827	NCI-H1437	NCI-H1975
EGF R	47	9	13	2
EphB6			8	
ErbB2	12	7	19	2
ErbB3			22	
FGF R3		8		
HGF R	7	7	14	
3D				
c-Ret				7
IGF-I R			4	7
Insulin R				6
2D				

MAP-Kinase Phosphoarray B				
	CaLu-1	HCC827	NCI-H1437	NCI-H1975
Akt3		6		4
CREB			4	4
ERK1			5	4
HSP27		5	4	4
MSK2		4		
p38 β		5		5
RSK1		5		
RSK2		5		
TOR		5		4
ERK2		2		
GSK-3 β			2	
p53			2	
3D				
2D				

Phospho-Kinase Phosphoarray C				
	CaLu-1	HCC827	NCI-H1437	NCI-H1975
Akt	7			
AMPK α 1		4		
AMPK α 2	6			3
Chk-2	6			
CREB	9		11	
eNOS			12	
Fgr			11	
Fyn		4		
GSK3 α/β	7			
Hck	6			
Lck	6			
Lyn	8			
MEK1/2			12	
MSK1/2		5	10	
p27		4	12	
p27			10	
p53		4		
Paxillin		4	18	
PLC γ -1			18	3
Pyk2			16	3
RSK1/2				3
Src	7			
STAT2				3
STAT3			11	
STAT5a		4		
STAT5b		4		
STAT6		6		3
TOR	6		8	3
Yes	7			
3D				
Akt				3
Chk-2				2
2D				

Apoptosis Array D				
	CaLu-1	HCC827	NCI-H1437	NCI-H1975
Bax		9		
Catalase	3	12	4	2
Cleaved Caspase-3		9		
Clusterin			5	
HSP27	3	10		2
HSP60	3			
HSP70				2
p27/Kip1	3		27	
p53 (S15)		10		
p53 (S46)		9		
SMAC/Diablo				2
TNF RI			6	
Caspase				2
Fas / TNFRSF6				2
Survivin				2
TRAIL R1 / DR4	2		2	2
3D				
2D				

Table 7: Fold changes of various phospho-protein patterns dependent of cultivation method of four lung cancer cell lines using phospho-antibody arrays.

Fold change of proteins involved in phosphorylation of RTK (**A**), MAPK (**B**) Phospho-Kinase (**C**) and Apoptosis (**D**) related proteins in 2D and 3D. The values in the 3D box indicate the fold changes of phosphorylation of a certain protein in comparison to 2D and vice versa.

4.4 Chemotherapeutics and Molecularly Targeted Treatment: Drug-Response of 3D-Cultured Tumor Cells in Comparison to 2D-Cultured Cells

We already demonstrated that 3D cell cultivation significantly influences gene expression and particularly phosphorylation of EGFR related signaling molecules. We hypothesized earlier that EGFR mutations and amplifications could be an indication for oncogene addiction of the mutation carrying cell lines. These mutations consequently alter cell morphology, behavior and could lead to an increased sensitivity to the EGFR inhibitor Gefitinib. The huge differences in the phosphorylation patterns between cells grown two-dimensionally and MCTOs might therefore be essential to be considered by evaluating drug candidates *in vitro*.

Since drug sensitivity and gene expression are strongly interdependent we determined the relationship between three-dimensional cultivation and therapeutic efficacy of anti-cancer agents. We used alamarBlue® reagent to determine cell viability dependent on the ability of living cells to reduce resazurin to resorufin producing red fluorescence (O'Brien J et al., 2000).

4.4.1 Chemotherapeutic Treatment

For monolayers cells were seeded in 96-well microtiter plates. Poly-HEMA coated 96-well microtiter plates were used for spheroid generation. For organoids the spheroids were embedded in Matrigel/collagen matrices. 96h after treatment, alamarBlue® was added and incubated for 6h at 37°C. Data were assessed using the PARADIGM™ Detection Platform and the appropriate detection cartridge at 595 nm.

Paclitaxel mediated cytotoxicity

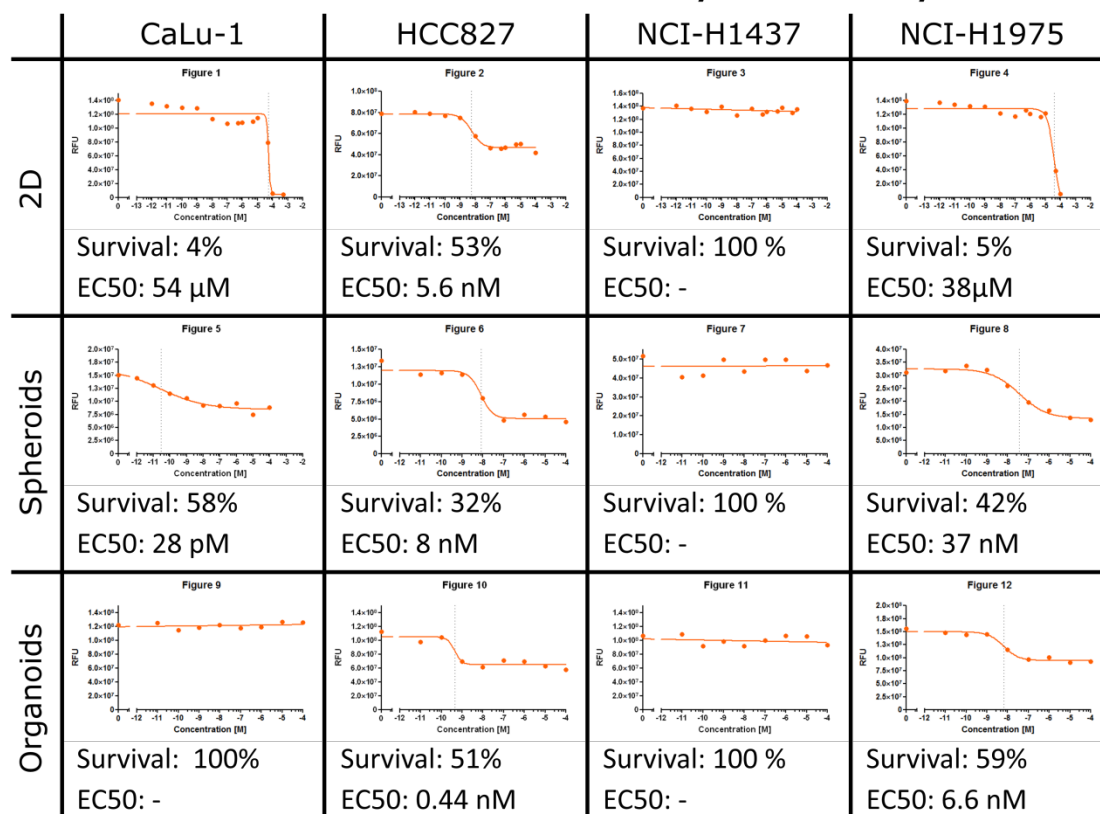


Figure 28: Response to paclitaxel of 3D-cultured tumor cells in comparison to 2D-cultured Cells.

Experiments were performed in 96-well microtiter plates. Cells were individually treated with different concentrations of paclitaxel for 96h. Cell survival was determined with alamarBlue® reagent (6h at 37°C). Data assessment by PARADIGM™ multimode reader. Curve fitting, graphing and calculation of EC50 values by the biostatistic software GraphPad Prism 5.

After paclitaxel treatment HCC827 showed similar drug responses independent on culturing method. However, the drug sensitivity slightly increased when the cells were cultured in 3D. CaLu-1 and NCI-H1975 cells grown in 2D showed a significant increase in survival compared MCTOs. This exemplifies the problematic nature of drug screening exclusively on monolayer cultures. Drug candidates that might have beneficial effects *in vivo* would not be identified (Figure 28).

Microscopic Examination of the Chemotherapeutic Effect of Paclitaxel on CaLu-1 microtumors | So far we have shown in the previous figure that paclitaxel treated CaLu-1 cells showed an increase in survival upon 3D cultivation compared to

2D-grown cells. AlamarBlue[®] reagent only allows detecting variances in the respiratory chain of cells being an indicator for cell viability. Due to that we performed additional phase-contrast imaging of paclitaxel treated CaLu-1 organoids to show phenotypic alterations due to this toxic agent.

After 96h of paclitaxel treatment, images of microtumors were acquired by phase-contrast microscopy. A classical morphology of adenocarcinoma is the cordlike infiltration pattern with Indian-file formation (Sternberg S, 2004). With increasing paclitaxel concentration these infiltration branches were significantly reduced (Figure 29). This might be due to paclitaxel mediated distortion of microtubule depolymerization which is important for matrix invasion. Despite this drastic effect on the invasive behavior, the survival of CaLu-1 organoids was not affected by paclitaxel as determined previously (Figure 28).

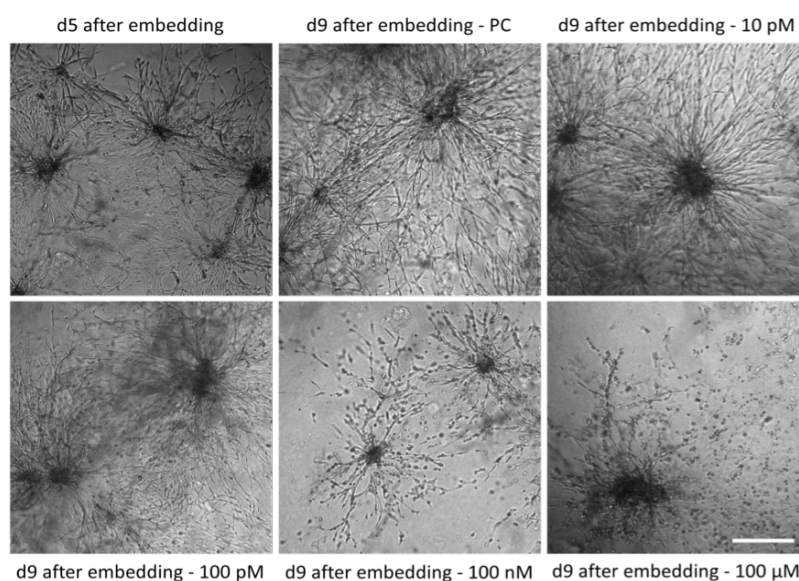


Figure 29: Therapeutic effect of the drug paclitaxel on the invasive potential of human lung cancer cell line CaLu-1 in Matrigel/collagen type 1 matrices (phase-contrast micrographs).

Spheroids were formed within 48h using poly-HEMA coated flat bottom 96-well plates. Organoids were embedded after 5 days in Matrigel/Collagen matrix consisting of 66% v/v Matrigel/collagen matrix diluted with growth medium. Phase-contrast micrographs show the paclitaxel dependent inhibition of invasion of CaLu-1 MCTOs after 96h of treatment. Bar, 200 μ m.

The Chemotherapeutic Effect of Cis-Diamminedichloroplatinum| Cisplatin (Cis-Diamminedichloroplatinum II, Cis-DDP) treatment elicited comparable cytotoxic effects on 2D and 3D cultured cells. Only at very high concentrations ($>100 \mu\text{M}$) we observed a probably drug-unrelated cytotoxicity resulting in cell death of all four cell lines (Figure 30). The different modes of action of paclitaxel and Cis-DDP could be an explanation for this effect. While paclitaxel interferes with microtubule formation Cis-DDP binds to the DNA and thereby elicits its genotoxic effect.

Cisplatin mediated cytotoxicity

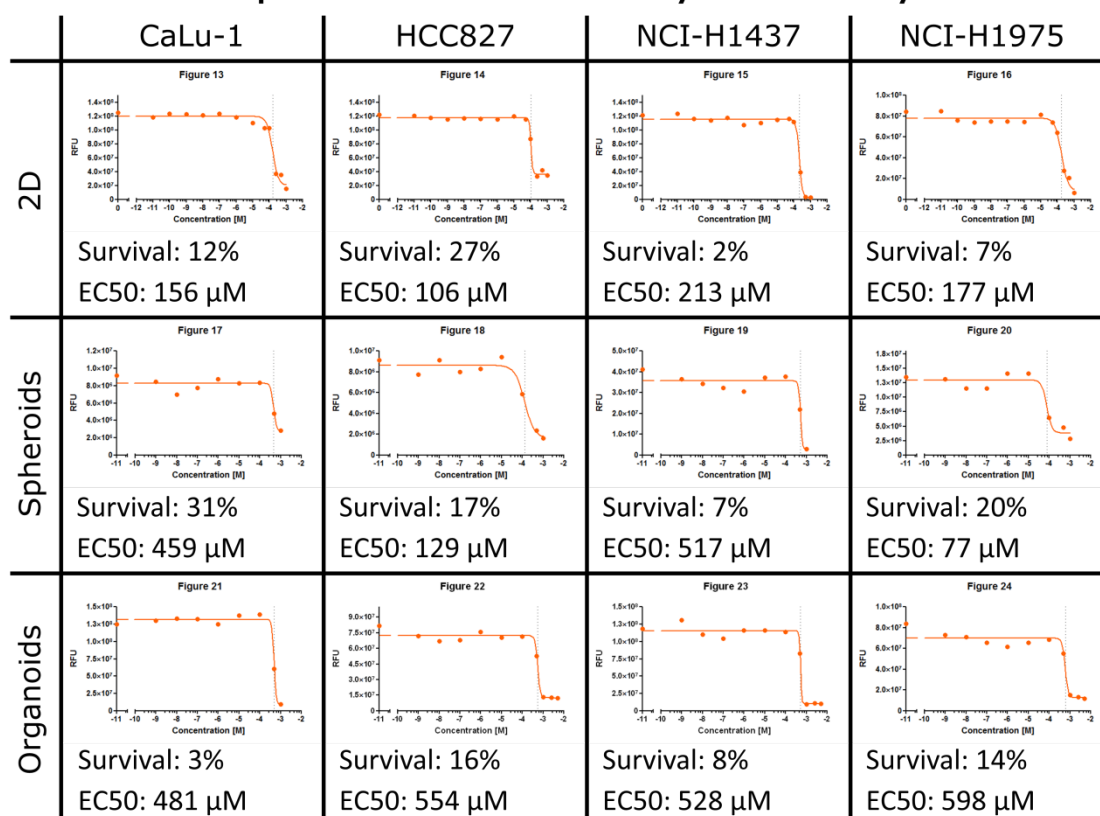


Figure 30: Response to Cisplatin of 3D-cultured tumor cells in comparison to 2D-cultured Cells.

Experiments were performed in 96-well microtiter plates. Cells were individually treated with different concentrations of Cisplatin for 96h. Cell survival was determined with alamarBlue® reagent (6h at 37°C). Data assessment by PARADIGM™ multimode reader. Curve fitting, graphing and calculation of EC50 values by the biostatistic software GraphPad Prism 5.

4.4.2 Targeted Therapies

Two of the four used cancer cell lines carry well described EGFR mutations while the other two cell lines express wild-type EGFRs. The impact of three-dimensionality on EGF signaling in these cells can therefore be evaluated in this model. To block EGF signaling the cells were treated with the EGFR inhibitor Gefitinib.

Cells (15000) were treated with Gefitinib for 96h and subsequently viable cells were quantified by alamarBlue® assay. Data were assessed using the PARADIGM™ Detection Platform and the appropriate detection cartridge at 595 nm (Figure 31).

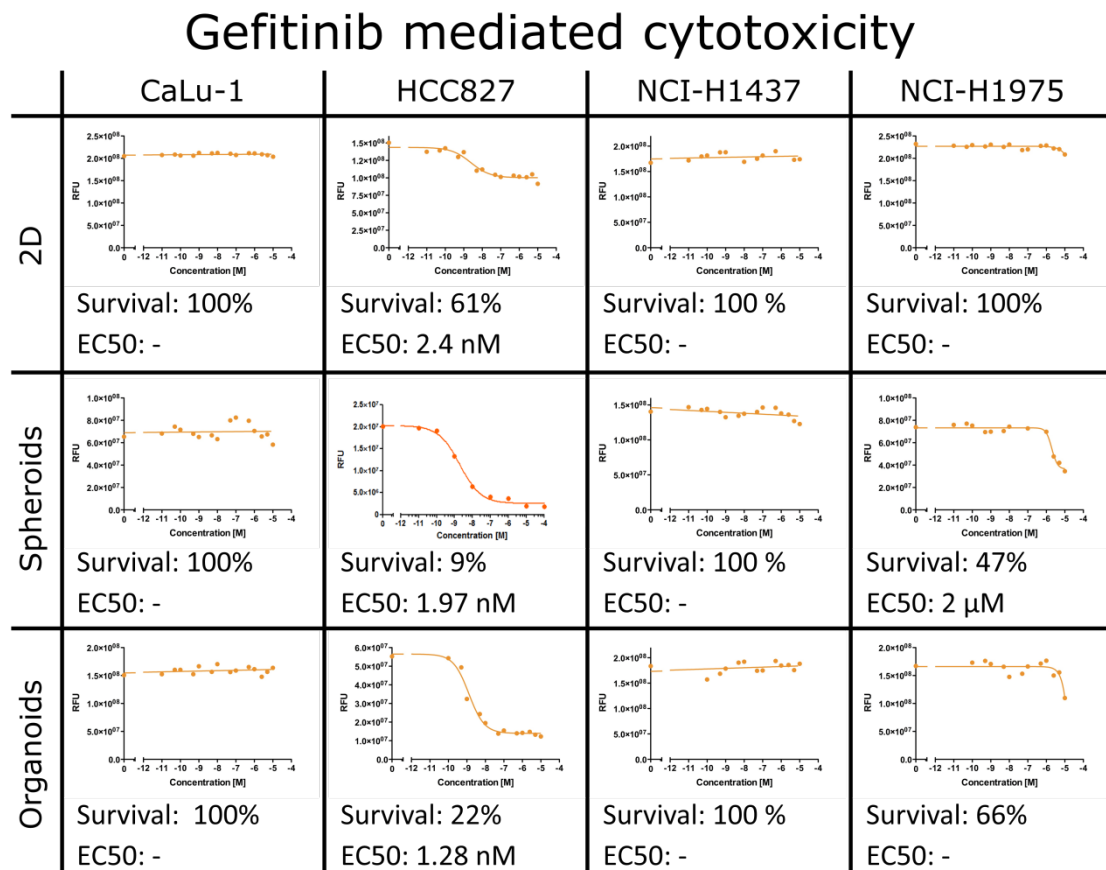


Figure 31: Response to Gefitinib of 3D-cultured cells in comparison to 2D-cultured cells.

Lung adenocarcinoma cells (CaLu-1, HCC827, NCI-H1437 and NCI-H1975) were grown as monolayer, spheroids or embedded organoids in 96-well microtiter plates. The cells were treated with different concentrations of Gefitinib for 96h. Viable cells were quantified with alamarBlue® reagent. Data assessment was performed by PARADIGM™ multimode reader. Curve fitting, graphing and calculation of EC50 values by the biostatistic software GraphPad Prism 5.

HCC827| Most significant is the decrease in survival at the highest drug concentration (10 μ M) when the cells are cultured as spheroids (9%) compared to organoids (22%) and monolayer culture (61%).

NCI-H1975| This cell line carries the drug sensitive point mutation, L858R, to HER1 inhibitors and a T790M point mutation which is associated with acquired drug resistance to Erlotinib or Gefitinib (Lynch TJ et al., 2004). Under conventional monolayer conditions NCI-H1975 cells were resistant to Gefitinib treatment. When grown three-dimensionally the cells' survival rates decreased down to 35% as spheroids and 5% as organoids.

Differential Proliferation of Lung Cancer Cell Lines in 2D and 3D

Many anti-cancer therapies are strongly dependent on the aberrantly up-regulated proliferation of malignant cells. Therefore we determined differential proliferation behavior of cells grown as a monolayer compared to multi cellular tumor spheroids (Figure 15). In both growth conditions NCI-H1437 cell line reached highest cell density followed by NCI-H1975 cell line. Grown two-dimensionally CaLu-1 and HCC827 cells reached a plateau after 12 days. After longer growth periods the proliferation curves of all cell lines cultured as spheroids started to flatten out and experienced a decline (CaLu-1, day 8; HCC827, day 6; NCI-H1437 and NCI-H1975 day 14). This was due to the onset of disintegration of the spheroids after prolonged incubation without being embedded. Therefore cell counting was stopped after 12 days (HCC827, CaLu-1) or 16 days (NCI-H1437, NCI-H1975). No increased apoptotic rates were observed in 2D and 3D cultures using in situ Annexin V assay (Kalod R, 2011).

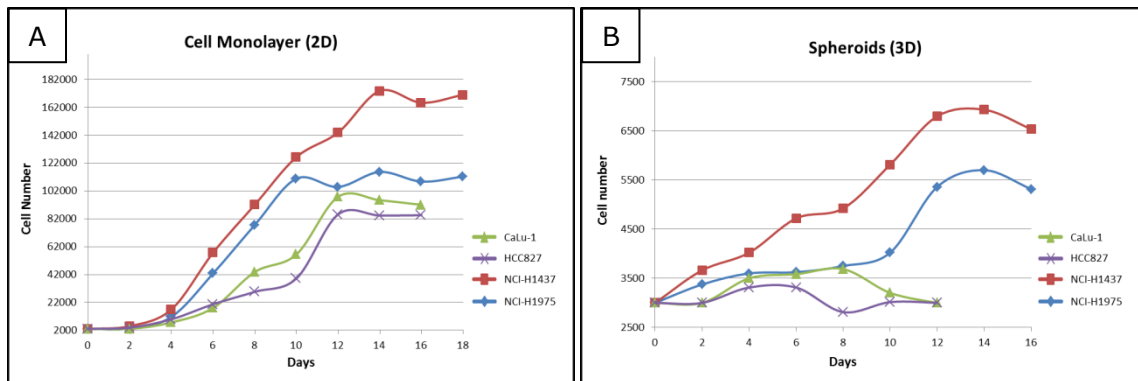


Figure 32: *In vitro* proliferation of a monolayer (A) or multi cellular tumor spheroids (B) of 4 human lung cancer cell lines.

Monolayer – 3000 cells were seeded per well of a flat bottom 96-well plate. Spheroids – using the methylcellulose method, 3000 cells were seeded per well of a round bottom 96-well plate. The cells and multicellular tumor spheroids (MCTS) were grown in defined growth media at 37°C and 5% CO₂ in a humidified atmosphere. In two-day-intervals the cells were harvested by trypsinization for determining cell number and viability (Neubauer counting chamber, Trypan Blue). Growth medium was replaced twice and in later stages of the experiment three times a week.

These findings indicate that the Gefitinib related cytotoxicity (Figure 31) is not interrelated with an increased proliferation but with EGFR oncogene addiction especially found in HCC827 cells.

4.5 Differentially Labeled Cell Types: Track the Cells' Fates in Heterotypic Co-Cultures

Apart from cancer cells a substantial part of solid tumors is made up by stromal cells such as fibroblasts, lymphocytes, macrophages, dendritic cells and other myeloid cells. The fine-tuned crosstalk between cancer cells and their surrounding microenvironment must be taken into consideration in order to evaluate drugs more reliable. Therefore it is essential to monitor the drug's effects on cancer cells and stromal cells likewise. To track the fate of each individual cell type within a heterotypic co-culture we lentivirally transduced cancer cells and stromal cells to stably express fluorescent proteins (LentiORF™ system, Thermo Fisher Scientific).

Three-Dimensional Co-Culture of Lung Cancer Cell Line HCC827 and Neonate Human Fibroblasts

Beside matrix related effects cellular crosstalk between tumor and stromal cells is a crucial factor in tumorigenesis. It is commonly accepted that stromal cells such as cancer-associated fibroblasts support tumor cells by fostering tumor invasion (Burdett E et al., 2010). Co-culture of lung cancer cells with fibroblasts reveals the supportive characteristic of stromal cells on the cancer cells.

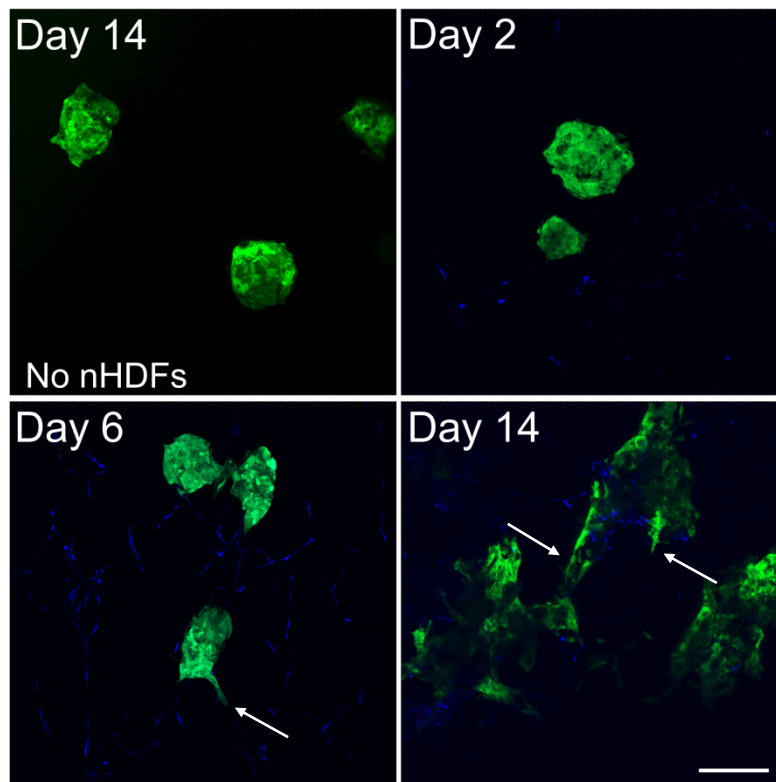


Figure 33: Co-culture of lentivirally transduced lung cancer cell line HCC827 and neonate human dermal fibroblasts (confocal laser scanning micrographs).

Both cell types were transduced using the lentiviral system LentiORF™. Tumor spheroids were generated using poly-HEMA coated round bottom 96-well plates. Organoids were embedded in 66% v/v Matrigel/collagen matrix in 96-well plates. 40.000 fibroblasts were co-embedded to generate heterotypic co-cultures. Confocal laser scanning micrographs were taken in 72h intervals. Images show the effect of fibroblasts on the invasive behavior of HCC827 MCTOs (arrows). Bar, 300 μ m.

The proliferative and invasive potential of lung cancer cell line HCC827 in Matrigel/collagen type 1 matrices was previously characterized (Figure 20). Embedded into Matrigel/collagen type 1 matrices, HCC827 organoids showed no significant invasive behavior. Upon co-cultivation with fibroblasts HCC827 cells showed high invasive potential. Already on day 6 first infiltration branches pointing towards fibroblasts are detectable (arrows, Figure 33). On day 12 the MCTO is almost completely deformed and interacts with the fibroblasts.

5 Discussion

The development of the first high-throughput cancer cell line screening program, the US National Cancer Institute 60 anti-cancer drug screen (NCI60), started in 1986. Since then the classical preclinical anti-cancer drug-screening strategies involved the growth of cancer cell lines as monolayers on tissue culture plastic combined with high-throughput screenings. Despite invaluable advances yielded by this methodology cells grown as monolayers remain poor predictors of drug efficacy and safety. It remained elusive whether a new compound will succeed as a potent anti-cancer drug in the clinic or ultimately fail. Reasons for that might be the absence of crucial environmental cues. Stromal cells, vasculature, 3D architecture, ECM and environmental stimuli such as growth factors or physico-chemical parameters (rigidity, fluid dynamics, etc.) found within tissues strongly impact tumorigenesis (Burdett E et al., 2010). As a consequence these environmental cues are obviously crucial elements of reliable cancer models.

Animal models are to date, a potent tool to study human cancer cells as subcutaneous, orthotopic or autochthonous xenografts (Killion J.J. et al., 1999; Talmadge J.E et al., 1986). Although these models provide tumor cells with a number of three-dimensional cues that are absent during monolayer growth they suffer from some inherent drawbacks. The complexity, difficulty, high expenditures of animal experiments and influences of the murine tumor stroma adversely affect the evaluation of drug efficacy. These drawbacks might relegate animal tumor models to a secondary role in the preclinical drug screening in near future.

To overcome shortcomings of classical cancer models in drug discovery, the development of complex three-dimensional cell-based cancer models gathered pace in the last years. The human tumor spheroid is the by far most widely used tissue-engineered tumor model. It might allow evaluating and identifying new anti-cancer agents more reliable than conventional cancer models. The aim of this study was to establish methods to generate 3D lung cancer models might be amenable to high-

throughput drug screening (HTS). Furthermore, we aimed to find differences in gene expression and phosphorylation of crucial signaling molecules due to 3D cultivation, and analyzed the efficacies of targeted drugs and chemotherapeutics in selected 3D models and conventional 2D cultures.

5.1 Characterization of Lung Cancer Cell Lines

5.1.1 Phase-contrast microscopy

We characterized four lung cancer cell lines in terms of morphology and marker protein expression when grown as monolayer, as spheroids and as multi cellular tumor organoids (MCTO) embedded in natural ECM scaffolds. We performed initial tests to choose an appropriate ECM to serve as a scaffold for the organoids. Matrigel™ and collagen type 1 are natural building blocks that can be used for the establishment of matrices that mimic the microenvironment of tumors. We used these components to analyze the differential growth behavior of multi cellular tumor organoids (MCTO) in matrices consisting of Matrigel™ or collagen type 1 and defined mixtures of those.

We observed that the cell lines featuring a rather undifferentiated phenotype when grown as monolayers had a higher invasive potential as MCTOs as well. NCI-H1975 and in particular CaLu-1 cells exhibited a more undifferentiated, mesenchymal phenotype and had an increased capability to invade the matrix. In contrast to that, the more differentiated, polarized carcinoma cell lines HCC827 and NCI-H1437 showed no significant invasive potential. We further showed that the invasive potential of CaLu-1 and NCI-H1975 cell line increased upon adding collagen type 1 to the matrix. Embedded in collagen type 1 matrices with collagen concentrations between 1.2 – 2.9 mg/mL these cell lines invaded the matrix most.

Pier P. Delsanto and his group reported that undernourishment of cells is increased with the gel concentration. The inhibitory effect of the matrix on the cell invasiveness at concentrations above 2.9 mg/mL collagen may be ascribable to the prolonged development of a suitable nutrient uptake of tumor cells within a stressed matrix (Delsanto PP et al., 2004).

5.1.2 Confocal laser scanning microscopy

Using immunofluorescent staining we could show that the expression of the epithelial differentiation marker E-cadherin was high in all used carcinoma cell lines grown as monolayers except CaLu-1. It is commonly accepted that in epithelial cells the downregulation of E-cadherin expression and concomitant *de novo* expression of vimentin is a hallmark of EMT (Liu T et al., 2012). We could demonstrate that CaLu-1 cells featured the highest expression levels of vimentin compared to all other investigated cell lines (Figure 13).

It has been consistently demonstrated that physiological cell signaling and related morphological and functional cellular changes rely crucially on the attachment of cells to surrounding three-dimensional mesh-like fibers (Cukierman E et al., 2002). Epithelial polarization is strongly interwoven with the formation of desmosomes and adherens junctions and the expression of E-cadherin. The loss of E-cadherin and the *de novo* expression of vimentin in epithelial cells is a hallmark for the transition to a mesenchymal phenotype (Thiery JP and Sleeman JP, 2006). This process, which is collectively termed epithelial-mesenchymal transition (EMT), is correlated with the loss of cell polarity and the detachment of cells from the position within the epithelial sheet during progression. Therefore EMT results in increased motility leading to tumor metastasis and a bad prognosis (Eger et al., 2004; Brabant G et al., 1993; Liu LK et al., 2010; Wirtz D et al., 2011). We could show that two differentiation markers that are known to control epithelial polarization are aberrantly regulated, resulting in different phenotypes among the four lung cancer cell lines (Figures 12, 13).

5.1.3 Differential Proliferation of Lung Cancer Cell Lines: 2D vs. Spheroids

Many anti-cancer therapies such as chemotherapies strongly depend on the sustained proliferation of malignant cells. Therefore we determined differential proliferation behavior of the cell lines grown as a monolayer compared to spheroids (Figure 15). It seems apparent that cell growth and proliferation are differentially regulated in cells grown as monolayers compared to 3D cultures. Freyer and Schor

reported that cells in the internal layers of MCTS experience an up to 3-fold size reduction (Freyer P and Schor PL, 1989). We also observed a significant reduction of cell size within spheroids and organoids. Interestingly, the cells within a MCTS proliferate approximately 5-times slower compared to the same cell line grown two-dimensionally. We determined the doubling time (DT) of NCI-H1437 cells grown as monolayer (47h) and grown as spheroids (244h). Consequentially, we assume that the DT of cells within MCTOs is even longer. It was reported by several groups that, at least for breast, colon and lung cancer, tumor volume doubling time (TVDT) *in vivo* is between 80-250 days (Friberg S and Mattson S, 1997; Spratt JS et al., 1996; Spratt JS and Spratt TL, 1964; Arai T et al., 1994).

After longer growth periods (8-16 days) the growth curves of all cell lines started to flatten out and declined. Since oxygen and nutrient supply is controlled by diffusion sufficient supply can only be ensured for cells residing in the spheroid's periphery (viable rim). Therefore in the spheroids' integrity might be lost after longer incubation periods.

5.2 Microarray Analyses reveal differential Gene Expression in 3D

The cell's pattern of gene expression determines its phenotype and function. Exploiting GeneChip human mapping assay technology (Affymetrix) provided us insight of differential gene expression in HCC827 and NCI-H1975 lung adenocarcinoma cell lines. We could show that gene expression patterns were significantly altered when the cells were grown three-dimensionally (Table 6 A, B).

In general we found that immunomodulatory proteins such as complement factor B and H are up-regulated in 3D. Lipocalin 2 which preserves Matrixmetalloproteinase (MMP)-9 activity and MMP-9 itself are up-regulated in 3D. It was reported that the increased expression of metalloproteinase (MMP)-9 promotes the invasive behavior of tumor cells (Paret C et al., 2010).

A remarkable number of genes encoding for small nucleolar RNAs (snoRNAs) are differentially expressed in MCTOs compared to monolayer cultured cells. These non-coding RNAs might promote oncogenesis as reviewed recently (Williams GT and Farzaneh F, 2012).

We currently perform pathway analyses using the Ingenuity software (Ingenuity Systems). This might help us to link deregulated genes with oncogenic pathways.

5.3 Three-Dimensionality alters Phosphorylation Patterns

Due to posttranslational modifications (PTMs) of proteins, a cell's proteome can consist of three fold more protein isoforms than the number of genes encoding them. These PTMs are essential to fine-tune signaling pathways of which many of those control proliferation and cell survival (Alberts B et al., 2008; Mann M and Jensen ON, 2003). A frequent reversible post-translational modification that plays a central role in tumorigenesis is phosphorylation. For this purpose it is essential to determine the phosphorylation patterns of certain protein panels known to be involved in tumorigenesis (Mann M and Jensen ON, 2003).

Cancer cells are strongly dependent on growth promoting signals for continuous oncogenesis. These signals are predominantly growth factors that bind receptor tyrosine kinase receptors e.g. epidermal growth factor receptor (EGFR) on the cell-membrane. It has been reported that non-small-cell lung cancer (NSCLC) frequently overexpresses the epidermal growth factor receptor (EGFR) encoded by ErbB-1 (Arteaga CL, 2002; Bunn PA Jr. and Franklin W, 2002). It was reported by different groups that various mutations in the EGFR tyrosine kinase domain lead to hyperactivation of the downstream signaling. Takayuki Kosaka reported in 2004 that more than 45% of lung cancer patients with EGFR mutations carry an E746-A750 deletion on exon 19 (Kosaka T et al., 2004). This most frequent exon 19 deletion,

that flanks the ATP-binding pocket, which is important for tyrosine kinase activity, leads to EGFR pathway hyperactivation. Therefore inhibitors targeting these receptors were recently developed and clinically approved. First promising data indicate that these targeted therapies may play a crucial role in the future treatment of cancer patients.

To investigate the therapeutic effect of targeted anti-cancer agents we used two cancer cell lines carrying EGFR mutations (HCC827, NCI-H1975) of known activities while the other two cell lines express wild-type EGFRs. The impact of three-dimensionality on the cells could therefore be evaluated in this model upon treating the cells with the EGFR inhibitor Gefitinib (Iressa). In addition, conventional chemotherapeutics were used to monitor their effect in the 3D model (Figures 31).

The 10-fold EGFR amplification, the E746-A750 deletion and the 9-fold higher pEGFR levels in HCC827 cell line could lead ultimately to oncogene addiction and hence to an increased sensitivity to RTKIs such as Gefitinib than wild-type. Using our three-dimensional model system we could show that this oncogene addiction in HCC827 was only detectable in 3D culture. While only 39% of 2D cultured cells died from Gefitinib treatment (10 μ M) 91% of spheroids and 78% of organoids died either by necrosis or apoptosis after 96h treatment (Figure 31).

Furthermore, a phosphorylated form of the epidermal growth factor receptor 1 (EGFR) was higher expressed (47-fold) in CaLu-1 organoids compared to 2D cell monolayer. EGFR stimulates downstream cell signaling cascades that influence beside others cell proliferation, migration and tumorigenesis. The remarkably high expression of this receptor in CaLu-1 MCTOs perfectly correlates with the highly invasive phenotype observed microscopically (Figures 15-17, 29).

Apart from EGFR, a phosphorylated form of receptor tyrosine kinase Tie-2 was higher expressed in organoids compared to conventional cell monolayer in (CaLu-1 7-fold; HCC827 21-fold). Tie-2 has been reported to play an important role in

vasculogenesis (Mitsuhashi N et al., 2003). EphA receptor 6 (EphA6) was up-regulated in organoids compared to conventional cell monolayer (CaLu-1 6-fold; HCC827 11-fold). Eph receptors are reported to influence down-regulation of cell-cell adhesion in cancer cells (Pasquale EB, 1997). Phosphorylated hepatocyte growth factor receptor (HGFR) was higher expressed in all cell lines except NCI-H1975 (CaLu-1 7-fold; HCC827 7-fold, NCI-H1437 14-fold). In cancer HGFRs have been implicated in angiogenesis, proliferation, survival, invasion and metastasis (Sattler M et al., 2011).

Taken together, we could demonstrate an increased phosphorylation of crucial signaling proteins associated in tumorigenesis when cells were cultured three-dimensionally. Since these PTMs are essential to regulate cancer-associated signaling pathways we conclude that

5.4 Chemotherapeutics and Molecularly Targeted Treatment

It may seem as if chemotherapy is still the treatment modality for the majority of cancers today. We tested two prominent members of this drug class, named paclitaxel and cisplatin (Cis-DDP), in our model and determined its efficacy compared to monolayer culture. In HC827 cells paclitaxel showed the best activity in organoids. Cis-DDP showed only at the highest concentrations therapeutic effects in our model (Figure 30). This might be ascribable to the lower overall proliferation rate (Figure 32) and by increased cell-cell contacts within the spheroids.

While the EC50 for paclitaxel treatment of CaLu-1 organoids was 54 μ M, even at concentrations below 100 nM a strong inhibitory effect on the invasion potential was visible (Figure 29). The cell line's typical infiltration branches that are usually formed were completely distorted at this concentration.

We hypothesized earlier that the 10-fold EGFR amplification and the E746-A750 deletion in HCC827 cells could be an indication for oncogene addition. This could

lead to an increased sensitivity to Gefitinib, an EGFR inhibitor. In fact this cell line showed highest sensitivity for Gefitinib. HCC827 organoids showed significant higher sensitivity for this RTKI compared to cells grown as monolayer. While 61% of two-dimensionally grown cells survived the highest RTKI concentration of 10 μ M only 91% of spheroids and 78% of organoids were dead (Figure 31).

Taken together we assume that not necessarily sustained proliferation but EGFR mediated survival in lung cancer cells, plays a pivotal role in the sensitivity for targeted therapies. By inhibiting EGFR signaling especially in association with PI3K action the absence of sustained pro-survival stimuli induces apoptosis. This case of oncogene addiction which was only observable in three-dimensional cultures depicts the physiology of this model system.

6 Conclusion

We established various methods to generate complex cell-based 3D cancer models on the basis of the human tumor spheroid methodology. 27 different lung, breast and colon cancer cell lines were phenotypically characterized within this model in several natural and synthetic matrices. In addition we constructed heterotypic co-cultures of lung cancer cells and stromal cells that stably express fluorescent proteins. Thereby we could demonstrate the influence of stromal cells on the invasive potential of cancer cells. Furthermore we implemented a standardized work flow for the production of 3D cancer models. Liquid handling robotics and high-content phenotype based screenings could enable HTS of millions of compounds within this 3D model system in the future.

We could show significant differences in gene expression and phosphorylation patterns in 2D and 3D cell cultures. Using phospho-proteome profiler antibody arrays we found a remarkable increase in RTK phosphorylation, especially in epidermal growth factor receptor (EGFR) signal transduction. In line with these findings the 3D models exhibited a significantly increased sensitivity to the targeted EGFR inhibitor Gefitinib (Iressa). Interestingly, in the clinic it could be demonstrated that the survival of lung cancer cells in cancer patients was strictly dependent on hyperactivated EGFR signaling (oncogene addiction). Treatment with Gefitinib induced apoptosis in cancer cells and caused the shrinkage of the tumors. Hence the organotypic models fully mimicked the in vivo situation whereas the 2D cultures were only marginally affected by the drug.

Taken together, we propose that 3D tumor models will play a major role in future drug discovery. However, much more work is clearly needed in order to fully exploit organotypic 3D models for basic and applied cancer research and personalized medicine.

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